

FORM PTO-1390
(REV 10/95)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. §371**

WEICKM 13

U.S. APPLICATION NO. (If known, see 37 CFR §1.5)

09/869709

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PCT/EP99/10480

29 DECEMBER 1999

PRIORITY DATE CLAIMED

30 DECEMBER 1998

TITLE OF INVENTION

METHOD FOR HIGH-THROUGHPUT-DETECTION OF RECEPTOR LIGAND INTERACTIONS

APPLICANT(S) FOR DO/EO/US


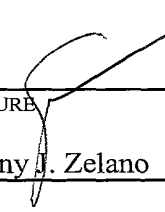
SIPPEL, Albrecht E., et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. §371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. §371(c)(2)).
- ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
- ☒ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. §§1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
 - ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

U.S. APPLICATION NO. (if known, see 37 CFR §1.5) 09/869709		INTERNATIONAL APPLICATION NO. PCT/EP99/10460		ATTORNEY'S DOCKET NUMBER WEICKM 13	
17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR §1.492 (a) (1) - (5)): Search Report has been prepared by the EPO or JPO..... \$860.00 International preliminary examination fee paid to USPTO (37 CFR §1.482)..... \$690.00 No international preliminary examination fee paid to USPTO (37 CFR §1.482) but international search fee paid to USPTO (37 CFR §1.445(a)(2))..... \$710.00 Neither international preliminary examination fee (37 CFR §1.482) nor international search fee (37 CFR §1.445(a)(2)) paid to USPTO..... \$1000.00 International preliminary examination fee paid to USPTO (37 CFR §1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 C.F.R. §1.492(e)). <input type="checkbox"/> 20 <input type="checkbox"/> 30					
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	70 - 20 =	50	x \$ 18.00	\$900.00	
Independent claims	1 - 3 =	0	x \$ 80.00	\$0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$ 270.00		
TOTAL OF ABOVE CALCULATIONS =				\$1,760.00	
Reduction of 1/2 for filing by small entity, if applicable. A Verified Small Entity Statement must also be filed (Note 37 C.F.R. §§1.9, 1.27, 1.28).					
SUBTOTAL =				\$1,760.00	
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 C.F.R. §1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30					
TOTAL NATIONAL FEE =				\$1,760.00	
Fee for recording the enclosed assignment (37 C.F.R. §1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§3.28, 3.31). \$40.00 per property.					
TOTAL FEES ENCLOSED =				\$1,760.00	
				Amount to be refunded:	
				charged:	
a. <input checked="" type="checkbox"/> A check in the amount of <u>\$1,760.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. <u>13-3402</u> in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>13-3402</u> . A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 C.F.R. §§1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. §1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO: Customer Number 23,599					
 23599 PATENT TRADEMARK OFFICE			SIGNATURE _____  Anthony J. Zelano NAME _____		
Filed: 2 JULY 2001 AJZ:jmm			<u>27,969</u> REGISTRATION NUMBER		

IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

International Application No. : PCT/EP99/10460
International Filing Date : 29 DECEMBER 1999
Priority Date(s) Claimed : 30 DECEMBER 1998
Applicant(s) (DO/EO/US) : SIPPEL, Albrecht, et al.

Title: METHOD FOR THE CELLULAR HIGH-THROUGHPUT-DETECTION OF
RECEPTOR LIGAND INTERACTIONS

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

SIR:

The claims of the above application were amended during the International Phase under Article 34. This Preliminary Amendment is based on the AMENDED claims.

Prior to calculating the national fee, and prior to examination in the National Phase of the above-identified International application, please amend as follows:

IN THE CLAIMS:

5. (Amended) A cell as claimed in claim 1, characterized in that the fusion protein required enzymatic modification before it can be bound to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and in that an enzymatic activity necessary for the enzymatic modification is activated only because of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section.

6. (Amended) A cell as claimed in claim 1, characterized in that the membrane receptor is a transmembrane receptor, an enzyme-coupled receptor, a G-protein-coupled receptor, a 7-transmembrane receptor or an odor receptor (or olfactorial receptor).

7. (Amended) A cell as claimed in claim 1, characterized in that the membrane receptor is a non-naturally occurring, synthetic membrane receptor.

9. (Amended) A cell as claimed in claim 1, characterized in that the ligand-binding section comprises the ligand-binding section of a transmembrane receptor, of a G-protein-coupled receptor, of a 7-transmembrane receptor, of an odor receptor (or olfactory receptor) or of a nuclear receptor, or is derived from the latter.

11. (Amended) A cell as claimed in claim 1, characterized in that the mediator section comprises the cytoplasmic part of a G-protein-coupled receptor, sections thereof or an amino acid sequence derived therefrom.

15. (Amended) A cell as claimed in claim 12, characterized in that the effector section of the fusion protein comprises the sequence of an active Ras protein.

18. (Amended) A cell as claimed in claim 1, characterized in that the mediator section is able, as a result of the ligand binding or, alternatively, lack of ligand binding to the ligand-binding section, to bind one or more adaptor proteins via which the effector protein or polypeptide which is capable of activating a Ras or Ras-like signal pathway in the cell, in the form of a fusion protein of an effector section with an adaptor protein or polypeptide section which makes binding possible to the component of the membrane via one or more of the adaptor proteins, can bind to the mediator section.

24. (Amended) A cell as claimed in claim 22, characterized in that the separate receptor-specific enzyme is a kinase and, in particular a tyrosine kinase.

25. (Amended) A cell as claimed in claim 18, characterized in that the adaptor proteins Gbr2 or Shc can be bound by the mediator section as a result of the ligand binding or, alternatively, lack of ligand binding to the ligand-binding section.

26. (Amended) A cell as claimed in claim 1, characterized in that the cell is a prokaryotic or eukaryotic cell.

28. (Amended) A cell as claimed in claim 1, characterized in that it is applied to a solid carrier.

30. (Amended) A cell as claimed in claim 1, characterized in that in the absence of the membrane receptor at least under certain conditions a Ras or Ras-like signal pathway in the cell cannot be activated.

34. (Amended) A cell as claimed in claim 31, characterized in that the lack of activatability of the Ras or Ras-like signal pathway in the absence of the membrane receptor above a particular temperature is derived from at least one mutation of a Ras protein intrinsic to the cell, which has the effect that the latter is incapable of functioning above the particular temperature.

35. (Amended) A *in vivo* assay for determining the suitability of a test substance as ligand for a ligand-binding section of a receptor, characterized by the following steps:

(a) contacting the test substance with cells as claimed in claim 30 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether activation of the Ras or Ras-like signal pathway has taken place, where detection of the activation of the Ras or Ras-like signal pathway indicates the ability of the test substance to bind to the ligand-binding section.

38. (Amended) A *in vivo* assay for determining the suitability of a test substance as ligand for a ligand-binding section of a receptor, characterized by the following steps:

(a) contacting the test substance with cells as claimed in claim 30 under

conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether activation of the Ras or Ras-like signal pathway has taken place,

(c) investigating cells employed in step (a) under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, for activation of the Ras or Ras-like signal pathway in the absence of the test substance, where detection of the activation of the Ras or Ras-like signal pathway in the absence of the test substance and the inactivity of the Ras or Ras-like signal pathway in the presence of the test substance indicates the ability of the test substance to bind to the ligand-binding section.

39. (Amended) An assay as claimed in claim 35, characterized in that the test substance is a naturally occurring substance and, in particular, an odorant, flavoring, peptide, peptide hormone, protein, in particular cytokine, growth factor, neurotransmitter, non-protein- or -peptide-like hormone and/or a vitamin.

40. (Amended) An assay as claimed in claim 35, characterized in that the test substance is a non-naturally occurring substance and, in particular, a synthetic derivative of a natural ligand or a poison, in particular dioxin.

42. (Amended) A screening method for unknown ligands of a particular receptor, characterized in that an assay method as claimed in claim 35 is employed for the screening.

43. (Amended) A *in vivo* assay for determining the detecting the presence of a ligand for a ligand-binding section of a receptor in a sample which possibly contains the latter, characterized by the following steps:

(a) contacting the sample with cells as claimed in claim 30 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether activation of the Ras or Ras-like signal pathway has taken place, where detection of the activation of the Ras or Ras-like signal pathway indicates the presence of a ligand for the ligand-binding section of a receptor in the sample.

46. (Amended) A *in vivo* assay for determining the presence of a ligand for a ligand-binding section of a receptor in a sample which possibly contains the latter, characterized by the following steps:

(a) contacting the sample with cells as claimed in claim 30 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether activation of the Ras or Ras-like signal pathway has taken place,

(c) investigating cells employed in step (a) under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, for activation of the Ras or Ras-like signal pathway in the absence of the sample, where detection of the activation of the Ras or Ras-like signal pathway in the absence of the sample and the inactivity of the Ras or Ras-like signal pathway in the presence of the sample indicates the presence of a ligand for the ligand-binding section of a receptor in the sample.

47. (Amended) A screening method for unknown ligands of a particular receptor in a sample, characterized in that an assay method as claimed in claim 43 is employed for the screening.

48. (Amended) A *in vivo* assay for the quantitative determination of the concentration of a ligand for a ligand-binding section of a receptor in a sample which contains the latter, characterized by the following steps:

(a) contacting an aliquot of the sample with cells as claimed in claim 30 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 30, is able to activate this Ras or Ras-like signal pathway,

(b) detecting quantitatively the extent of the activation of the Ras or Ras-like signal pathway by direct or indirect means,

(c) measuring the concentration of the ligand in the sample by comparing the measured extent of activation with corresponding values measured for known standard concentrations of the ligand.

51. (Amended) A *in vivo* assay for determining whether a compound is able to alter a binding activity of a ligand-binding section of a receptor in relation to a ligand, characterized by the following steps:

(a) contacting the ligand in the presence of the compound with cells as claimed in any of claim 30 under conditions with which in the absence of the membrane receptor the Ras or Ras-like signal pathway in the cells cannot be activated, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of or, alternatively, the lack of binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether and, where appropriate, to what extent activation of the Ras or Ras-like signal pathway takes place,

(c) comparing the result of the investigation in step (b) with a result of an investigation obtained when the assay is carried out in the absence of the compound.

55. (Amended) A *in vivo* assay for detecting whether a polypeptide or protein has a ligand-binding function of a receptor, characterized by the following steps:

(a) contacting cells as claimed in claim 30 with the ligand under conditions with which in the absence of the membrane receptor, defined in claim 30, a Ras or Ras-like signal pathway in the cells cannot be activated, where the ligand-binding section of the membrane receptor comprises the polypeptide or protein to be investigated or consists thereof, and where the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor, is able to activate the inactive Ras or Ras-like signal pathway,

(b) investigating whether an activation of the Ras or Ras-like signal pathway has taken place, where detection of the activation of the Ras or Ras-like signal pathway indicates that the ligand-binding section of the membrane receptor and, accordingly, the polypeptide or protein to be investigated has a ligand-binding function of a receptor.

57. (Amended) An assay as claimed in claim 55, where step (b) comprises detecting the activation of the Ras or Ras-like signal pathway via reporter gene expression which takes place where appropriate and only because of the activation, resulting from the activation of the Ras or Ras-like signal pathway, of a specific transcription factor, where detection of the expression of the reporter gene indicates the ligand-binding function of the ligand-binding section of the membrane receptor and, accordingly, of the polypeptide or protein to be investigated.

58. (Amended) An assay as claimed in claim 55, where in step (a) cells in which the inactive or inactivatable Ras or Ras-like signal pathway is a signal pathway which acts on the cell cycle and whose activation is essential for cell reproduction are employed, and step (b) comprises investigating whether the cells are capable of reproduction under said conditions, where detection of the ability of the cells to reproduce indicates the ligand-binding function of the ligand-binding section of the membrane receptor and, accordingly, of the polypeptide or protein to be investigated.

59. (Amended) A *in vivo* assay for determining whether a polypeptide or protein has ligand-binding function of a receptor, characterized by the following steps:

(a) contacting the cells as claimed in claim 30 with the ligand under conditions with which in the absence of the membrane receptor, as defined in claim 30, a Ras or Ras-like signal pathway in the cells cannot be activated, where the ligand-binding section of the membrane receptor comprises the polypeptide or protein to be investigated or consists thereof, and where the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor, is able to activate the inactive Ras or Ras-like signal pathway,

(b) investigating whether an activation of the Ras or Ras-like signal pathway has taken place,

(c) investigating cells as employed in step (a) under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, for activation of the Ras or Ras-like signal pathway in the absence of ligands, where a detection of the activation of the Ras or Ras-like signal pathway in the absence of the ligand and the inactivity of the Ras or Ras-like signal pathway in the presence of the ligand indicates that the ligand-binding section of the membrane receptor and, accordingly, the polypeptide or protein to be investigated has a ligand-binding function of a receptor.

60. (Amended) A kit for use in an assay as claimed in claim 35, which comprises cells as claimed in claim 35.

61. (Amended) A kit for use in an assay as claimed in claim 35, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

(a) cells in which at least under certain conditions a Ras or Ras-like signal pathway cannot be activated;

(b) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes a membrane receptor, as defined in claim 35, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of ligand to the ligand-binding section of the membrane receptor

is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

(c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

62. (Amended) A kit for use in an assay as claimed in claim 35, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

(a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;

(b) a nucleic acid vector which comprises, in suitable arrangement;

- a DNA section which encodes a membrane-localization signal of a membrane receptor, as defined in claim 35;

- a DNA section which encodes a mediator section of a membrane receptor, as defined in claim 35; and

- a suitably arranged insertion site for functional insertion of a DNA sequence which encodes a ligand-binding section, as defined in claim 35, where, after insertion of a DNA sequence for the ligand-binding section, the nucleic acid vector comprises a complete expressible gene for a membrane receptor, as defined in claim 35, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned

under (a);

(c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

63. (Amended) A kit for use in an assay as claimed in claim 55, which comprises cells as claimed in claim 55, where the membrane receptor, as defined in claim 55, present therein comprises a ligand-binding section comprising or consisting of a polypeptide or protein suspected of having a ligand-binding function of a receptor.

64. (Amended) A kit for use in an assay as claimed in claim 55, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

(a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;

(b) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes a membrane receptor, as defined in claim 55, where the ligand-binding section of the membrane receptor comprises a polypeptide or protein suspected of having a ligand-binding function of a receptor, or is formed therefrom, and the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

(c) a nucleic acid vector into which is expressibly inserted a DNA sequence which

encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

65. (Amended) A kit for use in an assay as claimed in claim 55, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

(a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;

(b) a nucleic acid vector which comprises, in suitable arrangement;

- a DNA section which encodes a membrane-localization signal of a membrane receptor, as defined in claim 55;

- a DNA section which encodes a mediator section of a membrane receptor, as defined in claim 55; and

- a suitably arranged insertion site for functional insertion of a DNA sequence which encodes a polypeptide or protein suspected of having a ligand-binding function of a receptor, where, after insertion of a DNA sequence for the ligand-binding section, the nucleic acid vector comprises a complete expressible gene for a membrane receptor, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section, formed from the polypeptide or protein suspected of having a ligand-binding function of a receptor, of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

(c) a nucleic acid vector into which is expressibly inserted a DNA sequence which

encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

66. (Amended) A kit as claimed in claim 60, in which the cells additionally contain a construct comprising a binding site for a transcription factor whose activation results from an activation of a specific Ras or Ras-like signal pathway whose activation is to be detected by the assay, a minimal promoter and a reporter gene functionally linked thereto, where the minimal promoter is activated as a result of binding of the activated transcription factor to its binding site.

67. (Amended) A kit as claimed in claim 60, characterized in that it additionally contains a transformation or transfection vector with a construct comprising a binding site for a transcription factor whose activation results from an activation of a specific Ras or Ras-like signal pathway whose activation is to be detected by the assay, a minimal promoter and a reporter gene functionally linked thereto, where the minimal promoter is activated transcription factor to its binding site.

68. (Amended) A kit as claimed in claim 60, characterized in that it additionally contains a transformation or transfection vector with a construct comprising a binding site for a transcription factor comprising a binding site for a transcription factor whose activation results from an activation of a specific Ras or Ras-like signal pathway whose activation is to be detected by the assay, a minimal promoter and an insertion site, suitably arranged for expression controlled by the minimal promoter, for insertion of a reporter gene, where the

minimal promoter is activated as a result of a binding of the activated transcription factor to its binding site.

69. (Amended) A kit as claimed in claim 60, which contains the cells immobilized or enclosed in microchambers of a solid carrier, in particular on biochips.

70. (Amended) A method for identifying polypeptides or proteins, in particular receptors, which have a ligand-binding function of a receptor, which comprises:

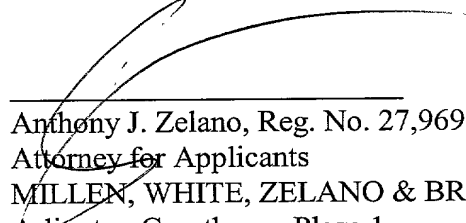
-preparing a cell as claimed in claim 1 with a membrane receptor having the features described in claim 1 and comprising the whole of such a polypeptide or protein or a part of such a polypeptide or protein which presumably contains the sequence sections essential for the ligand-binding function, and

-using this cell to carry out an *in vivo* assay method for detecting whether a polypeptide or protein has a ligand-binding function of a receptor, as claimed in claim 1.

REMARKS

The purpose of this Preliminary Amendment is to eliminate multiple dependent claims in order to avoid the additional fee. Applicants reserve the right to reintroduce claims to canceled combined subject matter.

Respectfully submitted,



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AJZ:jmm

FILED: 2 JULY 2001

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Claims 5-7, 9, 11, 15, 18, 24-26, 28, 30, 34-35, 38-40, 42-43, 46-48, 51, 55 and 57-70 have been amended as follows:

5. (Amended) A cell as claimed in ~~any of claims 1 to 4~~, characterized in that the fusion protein required enzymatic modification before it can be bound to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and in that an enzymatic activity necessary for the enzymatic modification is activated only because of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section.

6. (Amended) A cell as claimed in ~~any of claims 1 to 5~~, characterized in that the membrane receptor is a transmembrane receptor, an enzyme-coupled receptor, a G-protein-coupled receptor, a 7-transmembrane receptor or an odor receptor (or olfactorial receptor).

7. (Amended) A cell as claimed in ~~any of claims 1 to 5~~, characterized in that the membrane receptor is a non-naturally occurring, synthetic membrane receptor.

9. (Amended) A cell as claimed in ~~any of claims 1 to 7~~, characterized in that the ligand-binding section comprises the ligand-binding section of a transmembrane receptor, of a G-protein-coupled receptor, of a 7-transmembrane receptor, of an odor receptor (or olfactorial receptor) or of a nuclear receptor, or is derived from the latter.

11. (Amended) A cell as claimed in ~~any of claims 1 to 10~~, characterized in that the mediator section comprises the cytoplasmic part of a G-protein-coupled receptor, sections thereof or an amino acid sequence derived therefrom.

15. (Amended) A cell as claimed in ~~any of claims 12 to 14~~, characterized in that the effector section of the fusion protein comprises the sequence of an active Ras protein.

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18. (Amended) A cell as claimed in ~~any of claims 1 to 10~~, characterized in that the mediator section is able, as a result of the ligand binding or, alternatively, lack of ligand binding to the ligand-binding section, to bind one or more adaptor proteins via which the effector protein or polypeptide which is capable of activating a Ras or Ras-like signal pathway in the cell, in the form of a fusion protein of an effector section with an adaptor protein or polypeptide section which makes binding possible to the component of the membrane via one or more of the adaptor proteins, can bind to the mediator section.

24. (Amended) A cell as claimed in claim ~~22 or 23~~, characterized in that the separate receptor-specific enzyme is a kinase and, in particular a tyrosine kinase.

25. (Amended) A cell as claimed in ~~any of claims 18 to 24~~, characterized in that the adaptor proteins Gbr2 or Shc can be bound by the mediator section as a result of the ligand binding or, alternatively, lack of ligand binding to the ligand-binding section.

26. (Amended) A cell as claimed in ~~any of claims 1 to 25~~, characterized in that the cell is a prokaryotic or eukaryotic cell.

28. (Amended) A cell as claimed in ~~any of claims 1 to 27~~, characterized in that it is applied to a solid carrier.

30. (Amended) A cell as claimed in ~~any of claims 1 to 29~~, characterized in that in the absence of the membrane receptor at least under certain conditions a Ras or Ras-like signal pathway in the cell cannot be activated.

34. (Amended) A cell as claimed in claim ~~31 or 32~~, characterized in that the lack of activatability of the Ras or Ras-like signal pathway in the absence of the membrane receptor above a particular temperature is derived from at least one mutation of a Ras protein intrinsic to the cell, which has the effect that the latter is incapable of functioning above the particular temperature.

35. (Amended) A *in vivo* assay for determining the suitability of a test substance as ligand for a ligand-binding section of a receptor, characterized by the following steps:

(a) contacting the test substance with cells as claimed in ~~any of claims 30-34~~ 30 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim ~~1~~30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether activation of the Ras or Ras-like signal pathway has taken place, where detection of the activation of the Ras or Ras-like signal pathway indicates the ability of the test substance to bind to the ligand-binding section.

38. (Amended) A *in vivo* assay for determining the suitability of a test substance as ligand for a ligand-binding section of a receptor, characterized by the following steps:

(a) contacting the test substance with cells as claimed in ~~any of claims 30-34~~ 30 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim ~~1~~30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether activation of the Ras or Ras-like signal pathway has taken place,

(c) investigating cells employed in step (a) under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, for activation of the Ras or Ras-like signal pathway in the absence of the test substance, where detection of the activation of the Ras or Ras-like signal pathway in the absence of the test substance and the inactivity of the Ras or Ras-like signal pathway in the presence of the test substance indicates the ability of the test substance to bind to the ligand-binding section.

39. (Amended) An assay as claimed in ~~any of claims 35 to 38~~, characterized in that the test substance is a naturally occurring substance and, in particular, an odorant, flavoring, peptide, peptide hormone, protein, in particular cytokine, growth factor, neurotransmitter, non-protein- or -peptide-like hormone and/or a vitamin.

40. (Amended) An assay as claimed in ~~any of claims 35 to 38~~, characterized in that the test substance is a non-naturally occurring substance and, in particular, a synthetic derivative of a natural ligand or a poison, in particular dioxin.

42. (Amended) A screening method for unknown ligands of a particular receptor, characterized in that an assay method as claimed in ~~any of claims 35 to 38~~ is employed for the screening.

43. (Amended) A *in vivo* assay for determining the detecting the presence of a ligand for a ligand-binding section of a receptor in a sample which possibly contains the latter, characterized by the following steps:

(a) contacting the sample with cells as claimed in ~~any of claims 30-34~~ 30 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim ~~1~~30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether activation of the Ras or Ras-like signal pathway has taken place, where detection of the activation of the Ras or Ras-like signal pathway indicates the presence of a ligand for the ligand-binding section of a receptor in the sample.

46. (Amended) A *in vivo* assay for determining the presence of a ligand for a ligand-binding section of a receptor in a sample which possibly contains the latter, characterized by the following steps:

(a) contacting the sample with cells as claimed in ~~any of claims 30-34~~ 30 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the

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absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim ~~1~~30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether activation of the Ras or Ras-like signal pathway has taken place,

(c) investigating cells employed in step (a) under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, for activation of the Ras or Ras-like signal pathway in the absence of the sample, where detection of the activation of the Ras or Ras-like signal pathway in the absence of the sample and the inactivity of the Ras or Ras-like signal pathway in the presence of the sample indicates the presence of a ligand for the ligand-binding section of a receptor in the sample.

47. (Amended) A screening method for unknown ligands of a particular receptor in a sample, characterized in that an assay method as claimed in ~~any of claims 43 to 46~~ is employed for the screening.

48. (Amended) A *in vivo* assay for the quantitative determination of the concentration of a ligand for a ligand-binding section of a receptor in a sample which contains the latter, characterized by the following steps:

(a) contacting an aliquot of the sample with cells as claimed in ~~any of claims 30-34~~ 30 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim ~~1~~30, is able to activate this Ras or Ras-like signal pathway,

(b) detecting quantitatively the extent of the activation of the Ras or Ras-like signal pathway by direct or indirect means,

(c) measuring the concentration of the ligand in the sample by comparing the

measured extent of activation with corresponding values measured for known standard concentrations of the ligand.

51. (Amended) A *in vivo* assay for determining whether a compound is able to alter a binding activity of a ligand-binding section of a receptor in relation to a ligand, characterized by the following steps:

(a) contacting the ligand in the presence of the compound with cells as claimed in any of claims ~~30-34~~ 30 under conditions with which in the absence of the membrane receptor the Ras or Ras-like signal pathway in the cells cannot be activated, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of or, alternatively, the lack of binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim ~~1~~30, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether and, where appropriate, to what extent activation of the Ras or Ras-like signal pathway takes place,

(c) comparing the result of the investigation in step (b) with a result of an investigation obtained when the assay is carried out in the absence of the compound.

55. (Amended) A *in vivo* assay for detecting whether a polypeptide or protein has a ligand-binding function of a receptor, characterized by the following steps:

(a) contacting cells as claimed in any of claims ~~30-34~~ 30 with the ligand under conditions with which in the absence of the membrane receptor, defined in claim ~~1~~30, a Ras or Ras-like signal pathway in the cells cannot be activated, where the ligand-binding section of the membrane receptor comprises the polypeptide or protein to be investigated or consists thereof, and where the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor, is able to activate the inactive Ras or Ras-like signal pathway,

(b) investigating whether an activation of the Ras or Ras-like signal pathway has taken place, where detection of the activation of the Ras or Ras-like signal pathway indicates that the ligand-binding section of the membrane receptor and, accordingly, the polypeptide or protein to be investigated has a ligand-binding function of a receptor.

57. (Amended) An assay as claimed in claim 55-or-56, where step (b) comprises detecting the activation of the Ras or Ras-like signal pathway via reporter gene expression which takes place where appropriate and only because of the activation, resulting from the activation of the Ras or Ras-like signal pathway, of a specific transcription factor, where detection of the expression of the reporter gene indicates the ligand-binding function of the ligand-binding section of the membrane receptor and, accordingly, of the polypeptide or protein to be investigated.

58. (Amended) An assay as claimed in claim 55-or-56, where in step (a) cells in which the inactive or inactivatable Ras or Ras-like signal pathway is a signal pathway which acts on the cell cycle and whose activation is essential for cell reproduction are employed, and step (b) comprises investigating whether the cells are capable of reproduction under said conditions, where detection of the ability of the cells to reproduce indicates the ligand-binding function of the ligand-binding section of the membrane receptor and, accordingly, of the polypeptide or protein to be investigated.

59. (Amended) A *in vivo* assay for determining whether a polypeptide or protein has ligand-binding function of a receptor, characterized by the following steps:

(a) contacting the cells as claimed in ~~any of claims 30-34~~ 30 with the ligand under conditions with which in the absence of the membrane receptor, as defined in claim ~~30~~, a Ras or Ras-like signal pathway in the cells cannot be activated, where the ligand-binding section of the membrane receptor comprises the polypeptide or protein to be investigated or consists thereof, and where the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor, is able to activate the inactive Ras or Ras-like signal pathway,

(b) investigating whether an activation of the Ras or Ras-like signal pathway has taken place,

(c) investigating cells as employed in step (a) under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, for activation of the Ras or Ras-like signal pathway in the absence of ligands, where a detection of the activation of the Ras or Ras-like signal pathway in the absence of the ligand

and the inactivity of the Ras or Ras-like signal pathway in the presence of the ligand indicates that the ligand-binding section of the membrane receptor and, accordingly, the polypeptide or protein to be investigated has a ligand-binding function of a receptor.

60. (Amended) A kit for use in an assay as claimed in ~~any of claims 35 to 54~~, which comprises cells as claimed in ~~any of claims 30-34~~ 35.

61. (Amended) A kit for use in an assay as claimed in ~~any of claims 35 to 54~~, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

(a) cells in which at least under certain conditions a Ras or Ras-like signal pathway cannot be activated;

(b) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes a membrane receptor, as defined in claim ~~135~~, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

(c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

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62. (Amended) A kit for use in an assay as claimed in ~~any of claims 35 to 54~~, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

(a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;

(b) a nucleic acid vector which comprises, in suitable arrangement;

- a DNA section which encodes a membrane-localization signal of a membrane receptor, as defined in claim 135;

- a DNA section which encodes a mediator section of a membrane receptor, as defined in claim 135; and

- a suitably arranged insertion site for functional insertion of a DNA sequence which encodes a ligand-binding section, as defined in claim 135, where, after insertion of a DNA sequence for the ligand-binding section, the nucleic acid vector comprises a complete expressible gene for a membrane receptor, as defined in claim 135, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

(c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

63. (Amended) A kit for use in an assay as claimed in ~~any of claims 55 to 59~~, which comprises cells as claimed in ~~any of claims 30-34~~ 55, where the membrane receptor, as defined in claim 155, present therein comprises a ligand-binding section comprising or consisting of a polypeptide or protein suspected of having a ligand-binding function of a receptor.

64. (Amended) A kit for use in an assay as claimed in ~~any of claims 55 to 59~~, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

(a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;

(b) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes a membrane receptor, as defined in claim 155, where the ligand-binding section of the membrane receptor comprises a polypeptide or protein suspected of having a ligand-binding function of a receptor, or is formed therefrom, and the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

(c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

65. (Amended) A kit for use in an assay as claimed in ~~any of claims 55 to 59~~, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

- (a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;
- (b) a nucleic acid vector which comprises, in suitable arrangement;
 - a DNA section which encodes a membrane-localization signal of a membrane receptor, as defined in claim 155;
 - a DNA section which encodes a mediator section of a membrane receptor, as defined in claim 155; and
 - a suitably arranged insertion site for functional insertion of a DNA sequence which encodes a polypeptide or protein suspected of having a ligand-binding function of a receptor, where, after insertion of a DNA sequence for the ligand-binding section, the nucleic acid vector comprises a complete expressible gene for a membrane receptor, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section, formed from the polypeptide or protein suspected of having a ligand-binding function of a receptor, of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);
- (c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);
- (d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

66. (Amended) A kit as claimed in ~~any of claims 60 to 65~~, in which the cells additionally contain a construct comprising a binding site for a transcription factor whose activation results from an activation of a specific Ras or Ras-like signal pathway whose activation is to be detected by the assay, a minimal promoter and a reporter gene functionally linked thereto, where the minimal promoter is activated as a result of binding of the activated transcription factor to its binding site.

67. (Amended) A kit as claimed in ~~any of claims 60 to 65~~, characterized in that it additionally contains a transformation or transfection vector with a construct comprising a binding site for a transcription factor whose activation results from an activation of a specific Ras or Ras-like signal pathway whose activation is to be detected by the assay, a minimal promoter and a reporter gene functionally linked thereto, where the minimal promoter is activated transcription factor to its binding site.

68. (Amended) A kit as claimed in ~~any of claims 60 to 65~~, characterized in that it additionally contains a transformation or transfection vector with a construct comprising a binding site for a transcription factor comprising a binding site for a transcription factor whose activation results from an activation of a specific Ras or Ras-like signal pathway whose activation is to be detected by the assay, a minimal promoter and an insertion site, suitably arranged for expression controlled by the minimal promoter, for insertion of a reporter gene, where the minimal promoter is activated as a result of a binding of the activated transcription factor to its binding site.

69. (Amended) A kit as claimed in ~~any of claims 60 to 68~~, which contains the cells immobilized or enclosed in microchambers of a solid carrier, in particular on biochips.

70. (Amended) A method for identifying polypeptides or proteins, in particular receptors, which have a ligand-binding function of a receptor, which comprises:

-preparing a cell as claimed in claim 1 with a membrane receptor having the features described in claim 1 and comprising the whole of such a polypeptide or protein or a part of such a polypeptide or protein which presumably contains the sequence sections essential for

the ligand-binding function, and

-using this cell to carry out an *in vivo* assay method for detecting whether a polypeptide or protein has a ligand-binding function of a receptor, as claimed in ~~any of~~ claims 55 to ~~59~~ 1.

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Method for the cellular high-throughput detection of
receptor-ligand interactions

The invention relates to the area of molecular biology.

5 It relates in particular to assay methods which are
used to detect specific interactions between an
extracellular ligand and an, in particular, membrane-
associated receptor, and aims inter alia at finding
10 novel functional ligands for receptors and, where
appropriate, detecting a ligand-binding function which
is characteristic of, in particular, membrane-
associated receptors, in polypeptides or proteins
suspected of such a function. In this connection, the
invention also relates to membrane receptors, in
15 particular fusion proteins, nucleic acids which encode
these membrane receptors, in particular fusion
proteins, vectors which comprise these nucleic acids,
cells which comprise these membrane receptors, in
particular fusion proteins, and kits, all of which can
20 be employed for the assay methods of the invention or
in connection with the latter.

Cells, particularly cells in multicellular organisms,
are dependant on responding appropriately to
25 extracellular signals from their surroundings. Signal
target cells make use of membrane-associated receptor
proteins for specific signal reception. Binding of
extracellular ligand molecules to the extracellular
sections of the receptor (ligand-binding section) leads
30 to a conformation-mediated relaying of the signal
through the transmembrane region of the receptor to the
cytoplasmic section of the receptor in the interior of
the cell (mediator section). It is thus possible for
the ligand binding-dependant molecular chain to be
35 picked up by adaptor molecules on the cytoplasmic side
of the receptor and relayed to signal transduction
pathways inside the cell (Figure 1). The effects of
receptor-mediated signal transduction are rapid changes

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in cytoplasmic structures and processes altering gene activity and the replication or breakdown of genetic material in the cell nucleus.

5 Membrane receptors thus mediate their intracellular relaying of signals via the cytoplasmic mediator section of the receptor. Depending on the characteristics of this mediator section, the membrane receptors are divided into those where the relaying of
10 the signal by the mediator takes place via an enzymatic protein kinase reaction (enzyme-coupled receptors) or via so-called G proteins (G-protein-coupled receptors).

The enzyme-coupled receptors include those where the
15 enzymatically active mediator section is located directly in the same receptor protein molecule which also carries the ligand-binding section. A classic example of this type of receptor is the epidermal growth factor (EGF) receptor (Schlessinger and Ulrich,
20 1992). With other enzyme-coupled receptors, the enzymatically active protein section is located in a second protein subunit. Classic examples of this type are many cytokine receptors (Stahl and Yancopoulos, 1993). It is common to all of them that the protein
25 kinase activity of the mediator section of the receptor is strictly dependant on the binding of the ligand to the ligand-binding domain of the receptor. Many of the enzyme-coupled receptors transmit their signal via adaptor proteins and polypeptides to members of the ras
30 family of monomeric GTPases which are able, inter alia, to activate a serine/threonine phosphorylation cascade (Nishida and Gotoh, 1993).

With the G-protein-coupled receptors, the cytoplasmic
35 receptor configuration depending on ligand binding is picked up by a G-protein complex which consists of three subunits and which relays the signal by dissociation into an activated membrane-bound $\beta\gamma$ part and into an activated $G\alpha$ protein subunit. During this,

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the $G\alpha$ protein subunit exchanges its cofactor GTP which is characteristic of the inactive state for the cofactor GTP which is characteristic of the activated state and which, after GTP to GDP hydrolysis, reinactivates the $G\alpha$ protein subunit. A wide variety of signal transduction pathways is coupled to this cycle via the activated $\beta\gamma$ subunit and/or the activated α subunit of the G-protein complex as mediators. The G-protein-coupled receptors include the large family of 7-transmembrane receptors. These include, for example, the toxin receptors, the odor receptors, neurotransmitter receptors and many hundreds more (Dohlman et al., 1991, Leurs et al., 1998). Examples of adaptor proteins for the G-protein mediators are adenylate cyclases, phospholipase C and other G-protein-coupled receptor kinases, and certain ion channels.

Membrane receptor-mediated signal transductions control the crucial intracellular processes for survival of the cells, for growth and cell division, for cell differentiation and for cell death (apoptosis). Errors in the important control pathways induced by mutations frequently contribute to the development of disease, and in particular, of cancer. It is understandable why the ligand-receptor interaction is of great scientific, pharmaceutical and commercial interest because it represents the principal point of contact between extracellular surroundings and intracellular events in all living cells. It is possible with the aid of the ligand molecules to intervene from outside in a natural way in virtually all important intracellular processes and take countermeasures if there are faulty developments. The effect of many pharmaceuticals is based on their function as agonists or antagonists of naturally occurring ligands. It is understandable why there is great interest in being able to detect and study such receptor-ligand interactions at the molecular level.

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A number of methods for detecting membrane receptor-ligand interactions already exists. One of the methods makes use of the fact that many receptors have, because of their evolutionary relationship, very similar structures and their functional parts of proteins, called domains, can easily be interchanged and fused together in a new arrangement. It is thus possible for a wide variety of extracellular ligand-binding domains to be linked to specific cytosolic reporter domains in order to create certain standardizations in the detection system in cells (see, for example, US Patent 4859609). The disadvantage of this method derives from the need to construct functional recombinant receptor fusion proteins which respond selectively to the receptor-ligand interaction to be tested in cells.

Another approach to the study of membrane receptor-ligand interactions starts from unmodified wild-type receptors but makes use of antibodies directed against the specific configurational states of the cytosolic receptor domains in order to measure the ligand interactions.

A further approach detects the receptor-ligand interaction *ex vivo*, i.e. outside the living cell, by attaching either extracellular receptor domains or the ligand molecule to a matrix over which a solution containing the ligand or the receptor, respectively, then flows. In this case, the effort for obtaining and attaching each individual receptor or ligand to the appropriate matrix surface is enormous. Another problem in this connection is also the extrapolation of the results obtained to the conditions in the membrane of the living cells, because the cellular conditions may differ considerably from the *ex vivo* conditions. However, the most serious problem in this connection is the lack of possibility of access to the genetic information of the novel receptor variants detected in screens or high-throughput assays.

The object on which the invention is based is to provide alternative assays suitable for detecting in vivo specific interactions between a ligand and an in particular membrane-associated receptor, which has, inter alia, the advantages of being able to detect ligand-receptor interactions more quickly than possible in the prior art, and being capable of being carried out using cells which are simple to manipulate, such as prokaryotic cells or yeast cells, or else using ghost forms, lacking cell walls, of, in particular, yeasts. In addition, because of the design of the assay, there is always a direct possibility of access to the genetic information underlying a receptor variant.

Further objects of the invention comprise the provision of cells, kits, membrane receptors, in particular fusion proteins, nucleic acids and vectors, all of which can be employed for the assay methods of the invention or in conjunction with the latter.

The objects on which the invention is based are met by the assay methods, kits, cells, fusion proteins, nucleic acids and vectors defined in the claims.

The present invention is based on the following realizations:

1. With every membrane receptor, the binding of the ligand initiates a change of configurational state in the extracellular domain and/or a covalent modification in the cytosolic part.

2. For every membrane receptor there are cytosolic adaptor proteins which are near the membrane or membrane-associated and are able to recognize the ligand-specific configurational changes or activated as a consequence thereof.

3. The membrane localization of certain components of the signal pathways is necessary for activation of various ras and ras-like signal transduction pathways (Schlessinger, TIBS, 18: 273-275, 1993).

5

4.a) If a protein from the Ras family or a guanine nucleotide exchange factor (GEF) is provided as effector protein or polypeptide in such a way that it is able to bind as a result of ligand binding to the extracellular domain of a membrane receptor to one component of the membrane, for example to the intracellular section (= mediator section) of the membrane receptor or to adaptor proteins located near the membrane or associated with the membrane, for which purpose it is provided where appropriate as fusion protein whose additional protein portion, which can also be referred to as adaptor section, makes binding possible to such a component of the membrane, in the event of such a binding the Ras protein or the GEF undergoes membrane localization which is necessary for activation of a ras or ras-like signal transduction pathway.

4.b) An alternative possibility is for a protein from the Ras family or a guanine nucleotide exchange factor (GEF) to be provided as effector protein or polypeptide in such a way that it is able to bind only as a result of a lack of ligand binding to the extracellular domain of a membrane receptor to a component of the membrane, for example to the intracellular section (= mediator section) of the membrane receptor or to adaptor proteins located near the membrane or associated with the membrane, for which purpose it is provided where appropriate as fusion protein whose additional protein portion, which can also be referred to as adaptor section, makes binding possible to such a component of the membrane. Because of such a binding, which in this variant is made possible only when there is a lack of ligand binding to the extracellular domain of the

membrane receptor, the Ras protein or the GEF then undergoes membrane localization which is necessary for activation of a ras or ras-like signal transduction pathway.

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5. A result of such a membrane receptor-ligand interaction can be detected in eukaryotic and, where appropriate, also prokaryotic cells. Cells in which a ras or ras-like signal transduction pathway can be inactivated at least under certain conditions at the level of the Ras protein specific therefor or of a guanine nucleotide exchange factor specific for the Ras protein are known in the prior art. If the effector protein or polypeptide explained under 4. is able in such a cell to activate precisely this inactivated signal pathway, the result is a cell in which the ras or ras-like signal transduction pathway which is intrinsic to the cell and which is inactive at least under certain conditions can be activated by this Ras protein - although in the case of variant 4.a) only in the presence of a ligand for the extracellular ligand-binding section of the membrane receptor, and in the case of variant 4.b) only in the absence of a ligand for the extracellular ligand-binding section of the membrane receptor.

This results in a cell in which the or a particular ras signal transduction pathway can be activated only as a function of a ligand binding to the ligand-binding section of a membrane-associated receptor (variant 4.a)) or only when there is a lack of ligand binding to the ligand-binding section of a membrane-associated receptor (variant 4.b)). This cell makes it possible to establish an *in vivo* assay method which, on the basis of detecting an activation, which has taken place where appropriate, of the specific ras signal transduction pathway, where appropriate indirectly via specific effects detectable on or in the cell, such as cell

growth, makes it possible to detect interactions between such a receptor and a ligand specific therefor.

5 This invention therefore relates firstly to a cell which comprises a membrane receptor which comprises a ligand-binding section, a membrane-localization signal and a mediator section, and which is characterized in that only when a ligand binds to the ligand-binding section or, alternatively, only when binding of a
10 ligand to the ligand-binding section is lacking is a structural change, in particular a conformational change and/or an enzymatic modification, e.g. by phosphorylation or dephosphorylation, brought about with effects on the mediator section to result in
15 binding of an effector protein or polypeptide, which is capable of activating a Ras or Ras-like signal pathway in the cell, to a component of the cell membrane, and in particular to the mediator section of the receptor protein, where appropriate via other proteins or
20 polypeptides.

The membrane receptor may be a naturally occurring receptor such as, for example, a transmembrane receptor, an enzyme-coupled receptor, a G-protein-coupled receptor, a 7-transmembrane receptor or an odor
25 receptor (or olfactorial receptor). The membrane receptor may naturally occur in the cell or may originate from a cell system of a different type or else a virus and have been introduced into the cell by
30 transformation or transfection.

An alternative possibility is for the membrane receptor also to be a non-naturally occurring, synthetic receptor. One of this type preferably comprises
35 sections or domains which in turn each occur in nature, such as, for example, the amino acid sequence of the ligand-binding section of a transmembrane receptor, of an enzyme-coupled receptor, of a G-protein-coupled receptor, of a 7-transmembrane receptor or of an odor

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receptor (or olfactorial receptor), but also that of a naturally occurring nuclear receptor, such as a steroid receptor, orphan receptor, vitamin receptor, for example vitamin D receptor, thyroxin receptor or
5 retinoic acid receptor, or that of a naturally occurring viral receptor, in particular membrane receptor, or sequences derived therefrom by amino acid attachment, exchange, modification, insertion or deletion, but may also comprise, for example, sections
10 generated by molecular modeling. Such a membrane receptor may comprise, for example, a ligand-binding section and a membrane-localization signal, both of which originate from a particular protein from a particular organism, and a mediator section which
15 derives from a different protein and, where appropriate, also organism. In particular, the ligand-binding section and the mediator section derive from different proteins and, where appropriate, also organisms; the membrane-localization signal may also
20 correspondingly derive from a different protein and/or organism or else be generated synthetically. In addition, the ligand-binding section may comprise a non-naturally occurring, synthetic ligand-binding section, for example generated by molecular modeling,
25 with in particular initially only suspected ligand-binding function.

In preferred embodiments of this invention, the membrane-localization signal of the membrane receptor
30 comprises the amino acid sequence of a transmembrane domain as found, in particular, in transmembrane receptors, of a farnesylation signal, myristylation signal or prenylation signal or is derived therefrom for example by amino acid exchange, modification,
35 insertion or deletion.

In the region of the membrane-localization domain or as an additional sequence section which is, in particular, located at the N terminus it is also possible to

provide a signal sequence which, although it does not serve to anchor the membrane receptor in the membrane as such, has the effect that the membrane receptor is transported after expression thereof with high efficiency onto the cell membrane. The higher concentration, resulting therefrom, of membrane receptor in the immediate vicinity of the membrane results in a higher rate of incorporation of the membrane receptor into the cell membrane because of the membrane-localization domain. Such signal sequences are preferably used specifically suited to the cell type in which the membrane receptor is to be expressed because, for example, signal sequences effective in yeast are effective only with lower efficiency in mammalian cells, and vice versa. Examples of such signal sequences are signal sequences of GPCRs intrinsic to yeast or of invertase intrinsic to yeast (SUC2) for preferred use in membrane receptors which are to be expressed in yeast cells.

Accordingly, depending on the desired cell type it may also be sensible to replace a signal sequence intrinsic to the receptor by a signal sequence which is more effective in the desired cell type, for example when working in particular in yeast cells by replacing a signal sequence intrinsic to the receptor at the N terminus of a membrane receptor protein by the signal sequence of one of the G-protein-coupled receptors (GPCRs) intrinsic to yeast or a similar signal sequence which ensures that the receptor is anchored in active form in the cell membrane. In the case of receptor tyrosine kinases (RTKs) or receptor phosphatases it is possible, in particular for work in yeasts, to mention by way of example replacement of the signal sequence intrinsic to the receptor, where present, by the signal sequence with the invertase intrinsic to yeast (SUC2) or another signal sequence which optimizes the anchoring of the receptor in active form in the cell membrane.

As already mentioned, the effector protein or polypeptide which is able to activate a Ras or Ras-like signal pathway is a guanine nucleotide exchange factor (GEF), e.g. the CDC25 protein from *Saccharomyces cerevisiae* or an SOS protein from a mammal or an SOS-like protein from any organism, or an active protein from the Ras family, is derived from such factors or proteins, or has such a function in a section thereof.

10 In a particular embodiment which is explained in more detail hereinafter, the effector protein or polypeptide which is able to activate a Ras or Ras-like signal pathway is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide
15 which makes binding to a component of the membrane possible in the case of ligand binding to the ligand-binding section of the membrane receptor or, alternatively, only in the case of a lack of ligand binding to the ligand-binding section of the membrane
20 receptor, where appropriate via other proteins or polypeptides.

In another specific embodiment, the fusion protein requires enzymatic modification before it can be bound
25 to the component of the membrane, where appropriate via other proteins or polypeptides. In this case, an enzymatic activity necessary for the enzymatic modification is activated in the cell system of the invention only because of ligand binding to the ligand-binding section or, alternatively, only because of lack
30 of ligand binding to the ligand-binding section.

In all cases, as a result of the ligand binding to the ligand-binding section of the membrane receptor or,
35 alternatively, as a result of the lack of ligand binding to the ligand-binding section of the membrane receptor there is in the cells a translocation of the effector protein or polypeptide onto the cell membrane, which makes activation of the Ras or Ras-like signal

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pathway possible. The translocation is brought about because of the binding of the effector protein or polypeptide to a component of the membrane and, in a preferred embodiment of those possible, to the mediator section of the membrane receptor, where appropriate via other proteins or polypeptides. The binding of the effector protein or polypeptide to the component of the membrane is possible only when a ligand binds to the ligand-binding section of this membrane receptor or, alternatively, only when there is a lack of binding of a ligand to the ligand-binding section of this membrane receptor, because this brings about a structural change, in particular conformational change, with effects on the mediator section, to result in the effector protein or polypeptide binding, where appropriate via other proteins or polypeptides, to the component of the membrane, and in a preferred embodiment to precisely this mediator section.

This binding of the effector protein or polypeptide to a component of the membrane, where appropriate via other proteins or polypeptides, can take place in various ways depending on the particular membrane receptor and, in particular, on the particular mediator section of the membrane receptor:

1) In a first variant, the mediator section corresponds to the cytoplasmic part of a G-protein-coupled receptor. Alternatively, it comprises sections, which are essential for the properties explained hereinafter of this part of G-protein-coupled receptors, of such receptors or else sequences which are derived from said parts or sections for example by amino acid attachment, substitution, deletion, insertion and/or modification but which still have the properties to be explained of the initial sequences.

The membrane receptor comprising said mediator section can be, for example, a naturally occurring

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7-transmembrane receptor. These receptors comprise, for example, the toxin receptors, the odor receptors, neurotransmitter receptors and many others (Dohlman et al., 1991, Leurs et al., 1998).

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In addition, the membrane receptor may also be a synthetic receptor, e.g. with the cytoplasmic part of a particular G-protein-coupled receptor as mediator section and with the ligand-binding domain of another
10 G-protein-coupled receptor or else of a different type of receptor. In a specific embodiment of the invention, with synthetic receptors of this type the ligand-binding section brings about, on binding of ligand, the structural change, in particular conformational change,
15 naturally occurring in this case with G-protein-coupled receptors, with an effect on the mediator section, the cytoplasmic part of the receptor. Alternatively, other types of structural changes with effects detectable according to the invention on the mediator section of
20 the receptor are also conceivable on binding of ligands.

As already explained, with G-protein-coupled receptors the cytoplasmic receptor configuration depending on a
25 ligand binding is picked up by a G-protein complex which consists of three subunits and which relays the signal by dissociation into an activated membrane-bound $\beta\gamma$ part and activated, likewise membrane-bound, $G\alpha$ protein subunit. Activation of the $G\alpha$ protein subunit
30 is associated with replacement of the cofactor GDP characteristic of the inactive state by the cofactor GTP which is characteristic of the activated state and which reactivates the $G\alpha$ protein subunit after GTP to GDP hydrolysis.

35

The relaying of the signal from the G-protein activation, i.e. dissociation, or inactivation, i.e. reassociation, to the G-protein mediators, various signal transduction pathways which in turn act on the

metabolism, is mediated by so-called adaptor proteins. Adaptor proteins for G-protein mediators are, for example, adenylate cyclases, phospholipase C and other G-protein-coupled receptor kinases, and certain ion
5 channels. Some of these bind in particular to the activated $\beta\gamma$ part, which is still associated with the mediator section and therefore membrane-associated, of the G-protein, while others in turn bind to the activated $G\alpha$ protein subunit which has dissociated off
10 but is likewise still membrane-bound.

An effector protein or polypeptide translocation onto the membrane can now take place

- 15 - via mediation by the membrane-associated activated $\beta\gamma$ part of the G-protein (variant (a)),
- via mediation by the activated $G\alpha$ protein subunit which has dissociated off from the $\beta\gamma$ part of the G-protein but is still membrane-bound (variant (b)) or
20 - via the mediator section of the membrane receptor in its specific structure as present after binding of a ligand to the ligand-binding section of the membrane receptor or, alternatively, when there is a lack of binding of a ligand to the ligand-binding section of
25 the membrane receptor (variant (c)).

Variants (a) and (b):

- An effector protein or polypeptide translocation onto the membrane via mediation by the membrane-associated
30 activated $\beta\gamma$ part of the G-protein (variant (a)) or via mediation by the activated $G\alpha$ protein subunit which has dissociated off from the $\beta\gamma$ part of the G-protein but is still membrane-bound (variant (b)) can take place
-- through "binding" to the membrane-bound activated
35 $\beta\gamma$ part (variant (a)) or to the membrane-bound activated $G\alpha$ protein subunit which has dissociated off the G-protein (variant (b)) directly (alternative (i)),
-- via one of said adaptor proteins which are associated with the membrane-associated activated $\beta\gamma$

part of the G-protein (variant (a)) or with the membrane-bound activated G α protein subunit which has dissociated off the G-protein (variant (b)) (alternative (ii)) or

- 5 -- via a "binding" to a membrane-associated molecule which is in a specifically modified state through mediation by the activated $\beta\gamma$ part (variant (a)) or the activated G α protein subunit (variant (b)) of the G-protein (alternative (iii)).

10

- For alternative (i), the effector protein or polypeptide is provided in the cell for example in the form of a fusion protein of an effector section which can bring about activation of the Ras or Ras-like
15 signal pathway with an adaptor protein which interacts with the $\beta\gamma$ part of the G-protein after the α subunit has dissociated off (variant (a)) or with the activated G α protein subunit after dissociation off from the $\beta\gamma$ part of the G-protein (variant (b)), in particular with
20 a kinase or a phospholipase C, e.g. a GRK2 or GRK3 kinase (GRK = "G-protein coupled receptor kinase") or with a phosphatidylcholine phospholipase C.

- An alternative possibility for alternative (i) is for
25 the effector protein or polypeptide also to be provided in the form of a fusion protein of the effector section with an antibody which specifically recognizes the $\beta\gamma$ part of the G-protein only after the α subunit has dissociated off (variant (a)) or specifically
30 recognizes the activated G α protein subunit after dissociation off from the $\beta\gamma$ part of the G-protein (variant (b)).

- For alternative (ii) it is possible to provide the
35 effector protein or polypeptide inter alia as fusion protein whose adaptor section binds to an adaptor protein with, ordinarily, enzymatic activity, as have been detailed above by way of example specifically for an interaction with G-proteins, the adaptor protein

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being able to associate or interact specifically with the $\beta\gamma$ part of the G-protein only after the α subunit has dissociated off (variant (a)) or with the activated $G\alpha$ protein subunit only after dissociation off from the $\beta\gamma$ part of the G-protein (variant (b)). The adaptor section of the fusion protein may in this connection form, in particular, an antibody or binding protein which specifically recognizes and binds the adaptor protein.

In another case, the adaptor protein also has the property of an antibody or binding protein, in which case the adaptor protein recognizes either specifically the $\beta\gamma$ part of the G-protein only after the α subunit has dissociated off (variant (a)) or the activated $G\alpha$ protein subunit only after dissociation off from the $\beta\gamma$ part of the G-protein (variant (b)). In this situation, the adaptor section of the fusion protein usually has in particular the function of a so-called "secondary antibody" or anti-antibody which recognizes the so-called "primary" antibody associated with the mediator section.

For alternative (iii), use is made of the fact that a G-protein in the activated state can activate a phosphatidylinositol 3-kinase (PI3K) which in turn, as a result of the activation, generates so-called second messenger compounds by phosphorylation of the D-3 position of the inositol ring of phosphoinositides (e.g. $\text{PtdIns}(3,4)\text{P}_2$ (AKT) or $\text{PtdIns}(3,4,5)\text{P}_3$ (BTK)). These phosphoinositides are membrane-associated and, in their phosphorylated state, bind so-called Src homology (2) (SH2) and pleckstrin homology (PH) domains. For alternative (iii) accordingly the effector protein or polypeptide is provided in a particular embodiment in the form of a fusion protein of the effector section, which is able to bring about activation of the ras or ras-like signal pathway, with an Src homology (2) (SH2) domain or with a pleckstrin homology (PH) domain. The

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translocation of the effector protein or polypeptide in the form of the effector section accordingly takes place only when phosphorylation takes place to give the specifically mentioned phosphoinositides as a result of
5 G-protein activation, as a result of which the (SH2) or (PH) domains of the fusion protein associate with these phosphoinositides.

Variant c:

10 As explained, an effector protein or polypeptide translocation onto the membrane can alternatively also take place via the mediator section of the membrane receptor in its specific structure present after binding of a ligand to the ligand-binding section of
15 the membrane receptor or, alternatively, when there is a lack of binding of a ligand to the ligand-binding section of the membrane receptor, specifically
-- through direct "binding" to the mediator section in its specific structure present after binding or only
20 when there is an lack of binding of a ligand to the ligand-binding section of the membrane receptor (alternative (i)) or
-- via one or more adaptor protein(s) which are associated with the mediator section in its specific
25 structure present after binding or only when there is a lack of binding of a ligand to ligand-binding section of the membrane receptor (alternative (ii)).

For alternative (i), the effector protein or
30 polypeptide is provided in the cell for example in the form of a fusion protein of an effector section which is able to bring about activation of the Ras or Ras-like signal pathway with an adaptor protein which interacts with the mediator section of the membrane
35 receptor only in its specific structure present after binding of a ligand to the ligand-binding section of the membrane receptor or, alternatively, only if there is a lack of binding of a ligand to the ligand-binding

section of the membrane receptor, in particular an antibody or binding protein of appropriate specificity. For alternative (ii) of this variant, the effector protein or polypeptide can be provided in particular as fusion protein whose adaptor section binds to an adaptor protein which can associate or interact specifically with the mediator section of the membrane receptor only in its specific structure present after binding of a ligand to the ligand-binding section of the membrane receptor or, alternatively, only when there is a lack of binding of a ligand to the ligand-binding section of the membrane receptor. The adaptor section of the fusion protein can in this connection in particular form an antibody or binding protein which specifically recognizes and binds the adaptor protein. In the specific case where the adaptor protein also shows the property of an antibody, in which case the adaptor protein recognizes the mediator section of the membrane receptor specifically only in its specific structure present after binding or only when there is a lack of binding of a ligand to the ligand-binding section of the membrane receptor, the adaptor section of the fusion protein in this situation has the function of a so-called "secondary antibody" or anti-antibody which recognizes the so-called "primary" antibody associated with the mediator section.

2) In a second variant, the receptors chosen for the membrane receptor have mediator sections which, on binding of ligand to the ligand-binding section of the membrane receptor or, alternatively, only when there is a lack of binding of ligand to the ligand-binding section of the membrane receptor, are able to bind adaptor proteins which facilitate the binding of the effector protein or polypeptide.

The membrane receptors suitable for this purpose, which, where appropriate, also provide only the mediator section, are, in particular, enzyme-coupled

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receptors with intrinsic enzymatic activity and, in particular, with kinase activity, for example tyrosine kinase activity, serine/threonine kinase activity or phosphatase activity, which is localized in the cytoplasmic section of the receptor. The enzymatic activity of the mediator section in this case becomes active only on binding or, alternatively, only when there is a lack of binding of ligand to the ligand-binding domain of the membrane receptor as a result of the structural and, in particular, conformational change brought about by the (lack of) ligand binding. The enzymatic activity is moreover in certain embodiments also necessary for the binding, possible when there is ligand binding or, alternatively, when there is a lack of ligand binding, of the adaptor protein(s) to the mediator section, e.g. via an in some cases multiple phosphorylation of the mediator section (cf. Fig. 2a and 3) and/or else via a phosphorylation of adaptor protein(s). One example of a corresponding naturally occurring enzyme-coupled receptor is the epidermal growth factor receptor (EGFR), which has tyrosine kinase activity (cf. Fig. 3).

The membrane receptors which can be employed in this case may be, as for 1), naturally occurring receptors, receptors derived from such receptors, or else synthetic receptors which comprise, in particular, sections of diverse origin with the aforementioned functions such as membrane localization, ligand binding and mediator function. These sections may in turn derive from naturally occurring receptors or proteins of other types, or be derived from such according to the criteria already explained. In a specific embodiment of the invention, also with the synthetic receptors of this type the ligand-binding section brings about, on binding of ligand, the structural and, in particular conformational changes which occur naturally with enzyme-coupled receptors in this case and which have an effect on the mediator section and,

in particular, on the enzymatic activity localized in this section. However, in this case too, other types of structural changes with effects detectable according to the invention on the mediator section of the receptor and, in particular, on the enzymatic activity localized in this section are also conceivable as an alternative.

An effector protein or polypeptide translocation onto the membrane can now take place by binding of the effector protein or polypeptide to the adaptor protein which is membrane-associated because of binding to the mediator section (alternative (a)) or through use of a fusion protein which contains the effector protein or polypeptide fused to the adaptor protein (alternative (b)).

For alternative (a) it is possible in a particular embodiment to choose mediator sections of membrane receptors which, in the event of binding of ligand to their ligand-binding section or, alternatively, when there is a lack of binding of ligand to their ligand-binding section, bind adaptor proteins to which guanine nucleotide exchange factor (GEF) naturally binds. The epidermal growth factor receptor (EGFR) and the adaptor proteins Grb2 and Shc which are specific for its cytoplasmic or mediator section represent one example of such a membrane receptor. In turn there is binding to these adaptor proteins Grb2 and Shc, which bind to the mediator section of the EGFR only in the event of ligand binding to the ligand-binding section thereof, by a guanine nucleotide exchange factor which is able to activate a ras or ras-like signal pathway in a cell.

In another embodiment of alternative (a), however, it is possible for the effector protein or polypeptide also to be provided in the form of a fusion protein of an effector section which is able to activate a Ras or Ras-like signal pathway in a cell, and of a section having the property of an antibody or binding protein,

and the section having the property of an antibody or binding protein specifically recognizes and binds the adaptor protein(s) which, as a result of ligand binding or, alternatively, because of a *lack* of ligand binding to the ligand-binding section of the membrane receptor, are bound to the mediator section thereof.

In a specific variant of this embodiment, the adaptor protein is not a naturally occurring adaptor protein such as Grb2 or Shc, or an adaptor protein derived therefrom, but likewise has the property of an antibody, and the antibody adaptor protein, as just explained, recognizes and binds the mediator section of the membrane receptor only as a result of ligand binding to the ligand-binding section or, alternatively, as a result of a *lack* of ligand binding with a ligand-binding section thereof. In this case, the adaptor section of the fusion protein then has the function of a so-called "secondary antibody" or an anti-antibody which recognizes the so-called "primary" antibody associated with the mediator section.

In alternative (b), the effector protein is provided in the form of a fusion protein which contains the effector section fused to the adaptor protein. Binding of the adaptor protein section of the fusion protein to the mediator section of a membrane receptor as a result of ligand binding or, alternatively, of a *lack* of ligand binding to the ligand-binding section thereof leads via the covalent linkage within the fusion protein to the effector section also being guided onto the membrane, and it is able to exert its effect there for activation of a Ras or Ras-like signal pathway.

One example of this variant (b) is a fusion protein which comprises as adaptor section a Gbr2 or Shc protein fused to a preferably constitutively active ras protein, for example the human constitutively active ras protein (Haras, L61) or to a functional GEF as

effector section. Such a fusion protein is employed, for example, again in conjunction with an EGFR membrane receptor or with a receptor which comprises the cytoplasmic part thereof, in which case the cytoplasmic or mediator section of the EGFR interacts with the Gbr2 or Shc portion of the fusion protein on binding of ligand and thereby guides the effector section onto the membrane (cf. also Fig. 2a, 3).

Another example of this variant (b) is a fusion protein which has as adaptor section an antibody or a binding protein which recognizes and binds specifically the mediator section of the membrane receptor only in its structure/conformation of the binding of a ligand to the ligand-binding section or, alternatively, when there is a lack of binding of a ligand to the ligand-binding section.

3) The third variant is similar to the second variant but the enzyme-coupled receptor employed as membrane receptor or used to form the mediator section of the membrane receptor does not have intrinsic enzymatic activity activated on ligand binding or, alternatively, when there is a lack of ligand binding but, on the contrary, activates, strictly dependent on its own activation by ligand binding or lack of ligand binding, a separate receptor-specific enzyme which is located, for example, on a separate subunit. Various cytokine receptors can be mentioned as examples of such a receptor type.

This separate receptor-specific enzyme may also, in a particular embodiment, be heterologous to the cell. The separate receptor-specific enzyme is preferably likewise a kinase and, in particular, a tyrosine kinase. Once again, the kinase activity preferably leads to phosphorylation of the mediator section, i.e. the cytoplasmic section of the membrane receptor and

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thus has an additional effect on the binding of an adaptor protein specific for the mediator section.

Otherwise, the same applies as for the membrane
5 receptor of variant 2) i.e. the latter can be a naturally occurring membrane receptor, a membrane receptor derived from such a membrane receptor, or a synthetic membrane receptor whose various domains or sections originate from different sources which, where
10 appropriate, may also be derived from naturally occurring sequences.

As with variant 2), it is also possible in this case for the effector protein or polypeptide translocation
15 onto the membrane to take place through binding of the effector protein or polypeptide to an adaptor protein which is membrane-associated because of binding to the mediator section (alternative (a)) or by use of a fusion protein which contains the effector protein or
20 polypeptide fused to the adaptor protein (alternative (b)).

It is also possible in this case to mention Grb2 or Shc as examples of possible adaptor proteins which can be
25 employed, where appropriate also as fusion proteins with an effector section which is able to activate the Ras or Ras-like signal pathway, but also antibodies or other types of binding proteins which recognize and bind specifically the mediator section of the membrane
30 receptor only in its structure/conformation after binding of a ligand to the ligand-binding section or, alternatively, only when there is a lack of binding of a ligand to the ligand-binding section, and can be employed in the form of fusion proteins with an
35 effector section. Alternatively, the antibody adaptor protein or binding protein adaptor protein comprises no effector function but is in turn recognized and bound by a fusion protein with an effector section and an adaptor section with the function in particular of a

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so-called "secondary antibody" or anti-antibody which recognizes the so-called "primary" antibody (antibody adaptor protein) associated with the mediator section, via this adaptor section.

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4) In another, fourth variant, the membrane receptor employed is a fusion protein which has within its sequence, in particular at or in the region of the C terminus, a so-called tag or epitope which is accessible for recognition by an antibody specific therefor or a binding protein specific therefor only on binding of ligand to the ligand-binding section, or alternatively, only when there is a lack of binding of ligand to the ligand-binding section. Examples of suitable tags or epitopes which can be mentioned are a Myc tag, His tag, hemagglutinin epitope and the like.

The tag or the epitope may be a part of the mediator section but may also be adjacent thereto or separated therefrom by an amino acid sequence section. However, the accessibility thereof is made possible for a specific antibody or a specific binding protein only because of conformational changes, where appropriate in combination with enzymatic activity, as a result of binding of ligand to the ligand-binding section of the membrane receptor or, alternatively, because of dissociation of ligand off from the ligand-binding section of the membrane receptor, i.e. in the latter variant when there is a lack of ligand binding. A tag or epitope can thus be attached to all the receptor types mentioned, that is to say a G-protein-coupled receptor (GPCR) as well as to receptor tyrosine kinases or receptor phosphatases, provided that the criteria explained above concerning the accessibility of the tag or epitope are met for an antibody or a specific binding protein.

The effector protein is in this case provided as fusion protein which comprises the effector section and an

antibody specific for the tag or the epitope, or a binding protein specific for the tag or the epitope. If the tag or the epitope becomes accessible as a result of ligand binding to the ligand-binding section or, alternatively, as a result of a lack of ligand binding and effects resulting therefrom on the mediator section for the antibody present in the fusion protein or the binding protein present in the fusion protein, the effector protein or polypeptide is translocated onto the membrane through the tag/epitope-antibody/binding protein reaction.

Thus, when the explained conformational preconditions are met, this fourth variant can be employed most widely because it does not otherwise have to take account of the specific requirements of naturally occurring mediator sections such as those of G-protein-coupled receptors, receptor tyrosine kinases or receptor phosphatases, or synthetic mediator sections derived therefrom. In addition, the necessity to provide as effector protein only a fusion protein with effector section and antibody or binding protein sections specific for the tag or epitope brings about a considerable simplification in the method for preparing cells of the invention and of the assay methods which are described in detail hereinafter and which make use of these cells.

If it is intended to carry out with the aid of the described cells the assays of the invention which, as explained in detail hereinafter, specifically detect the interaction of ligands with the ligand-binding section of membrane receptors, it is self-evident that the event of translocation of the effector protein or polypeptide onto the cell membrane must occur only if there is a ligand-ligand-binding section interaction or, alternatively, if there is a lack of a ligand-ligand-binding section interaction on the membrane receptor specifically to be investigated. This means

that association of the effector protein or polypeptide with membrane-associated cell components which (in the absence of the membrane receptor and of the cell components cooperating specifically and exclusively with the latter for signal transduction) naturally occur in these cells must be precluded irrespective of the activation or modification state of the cell components, generally or when certain particular cultivation conditions are chosen for the cells. The precondition for this is that all components directly and indirectly involved in the translocation of the effector protein or polypeptide onto the cell membrane can, generally or when these particular cultivation conditions are chosen, only as a result of the activation of the membrane receptor specifically to be tested, because of the binding of a ligand to the ligand-binding section or, alternatively, because of the lack of ligand binding to the ligand-binding section, and more accurately only as a result of the structural change in the mediator section caused by this activation, be made able to mediate or bring about the binding of the effector protein or polypeptide to a component of the cell membrane.

If it is intended to test a membrane receptor naturally occurring in a cell in a test, it must be ensured that the mediator section of this membrane receptor occurs in the cell only in conjunction with the latter and that the adaptor and facilitator proteins cooperating therewith can interact only with this mediator section or only because of activation of this mediator section in such a way that translocation of the effector protein or polypeptide onto the cell membrane eventually occurs.

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An alternative possibility is for a cell to be selected as assay cell in which, before introduction of the genetic information for the membrane receptor and the adaptor, facilitator and/or effector proteins or

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polypeptides which are required where appropriate, none of these components naturally occurs, that is to say are heterologous to this initial cell, and no components of the same specificity and, where appropriate, activity which might replace the latter are intrinsically present in the cell either; in relation to the membrane receptor, this may also apply only to the mediator section. Or care must be taken that an initial cell which in an original state once contained these components, in relation to the membrane receptor where appropriate also only the specific mediator section, no longer contains these components because of genetic or other modification before introduction of the genetic information for the membrane receptor and the adaptor, facilitator and/or effector proteins or polypeptides which are required where appropriate. Explained comprehensively, this relates at least to the following components:

- the mediator section,
- where appropriate the adaptor proteins or adaptor protein sections cooperating therewith where binding of the effector protein or polypeptide takes place to the latter or via the latter, and
- for example in the case of alternative 1)c) explained herein, where appropriate a facilitator component which need not be directly spatially associated with the mediator section of the membrane receptor but is, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section, activated or modified by the latter and, for this reason, facilitates a secondary event on another component of the cell membrane, as a result of which the effector protein or polypeptide specifically binds to this component of the cell membrane, which is not associated with the mediator section of the membrane receptor; alternatively, said secondary event in the cell may be facilitated only if there is ligand binding or, alternatively, lack of

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ligand binding of the ligand-binding section of the membrane receptor. Thus, in the latter case, no interaction partners for these facilitators which are able to exert an activation effect corresponding to the membrane receptor on the facilitator(s) must be present in the cell.

Accordingly, in some embodiments of the invention, the expression of certain types of proteins and fusion proteins in a suitable cell system which does not naturally contain these proteins and fusion proteins is an essential aspect. Depending on the membrane receptor employed and, in particular, depending on the mechanism which is determined by the mediator section of the membrane receptor and which eventually leads to translocation of the effector protein or polypeptide onto the membrane, it may be necessary to express one or more of the aforementioned proteins in a cell which does not naturally contain the latter. These proteins can be expressed in their naturally occurring form, if there is one such, or in the form of fusion proteins.

It is possible in this connection for the gene constructs which encoded these proteins or fusion proteins to be present in the cell chromosomally, i.e. integrated into the chromosome, or extrachromosomally, as constituent of an episome, in particular plasmid.

A first fusion protein of this type may be a membrane receptor as explained above, with which in particular and, where appropriate, also exclusively the mediator section is heterologous to the cell.

Another one of these fusion proteins provides the effector protein or polypeptide in a particular form and consists of domains or parts which combine the following functions:

- i) the membrane-associated specific interaction of an adaptor section of the fusion protein with a

component of the membrane and, in a particular embodiment, with the mediator section of the receptor as a function of the extracellular ligand binding or, alternatively, of the lack of extracellular ligand binding (adaptor function);

5 ii) the possibility of activating a ras or ras-like signal transduction pathway as a function of the membrane localization determined by the adaptor section on the basis of an effector section fused to the adaptor section in the fusion protein

10 (effector function).

The former function has the effect that the adaptor-effector fusion protein reaches the membrane and thus the site of action of that part of the fusion protein which is responsible for the second function only when a ligand has bound to the usually extracellular part, i.e. the ligand-binding section, of the receptor or, alternatively, when no such ligand binding is present.

15

20 In the former case, the receptor without ligand binding is present in a configuration which is unable to bind the adaptor section directly, or is unable to induce a membrane-associated subsequent process via a mediator which the adaptor section would be able to recognize.

25 In the case of ligand binding, the cytosolic part of the receptor in this variant then assumes a configuration which the adaptor part is able to recognize directly, or it induces a subsequent process leading to a membrane-associated protein configuration of other mediator proteins which the adaptor is able to recognize. Thus, in the absence of ligand binding, the adaptor-effector fusion protein in the cytosol is not present at the site of action of the effector section.

30

35 Only when the effector section is in a membrane-associated orientation can it act as signal transduction component in the ras or ras-like signal transduction pathway (Fig. 2a and b).

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In the latter case, the receptor is, when there is binding of ligand, present in a configuration which is unable to bind directly the adaptor section or is unable to induce a membrane-associated subsequent process via a mediator which the adaptor section would be able to recognize. When the ligand dissociates off, or if ligand binding is lacking, the cytosolic part of the receptor in this variant then assumes a configuration which the adaptor part is able to recognize directly, or it induces a subsequent process which leads to a membrane-associated protein configuration of other mediator proteins which the adaptor is able to recognize. Thus, in this variant, the adaptor-effector fusion protein is not present at the site of action of the effector section in the cytosol when there is ligand binding. Only when the effector section is in a membrane-associated orientation can it act as signal transduction component in the ras or ras-like signal transduction pathway.

Such an activation can, as explained in more detail hereinafter, be detected from phenotypical changes (e.g. growth or gene or reporter gene activity) in the cell, it being necessary in the first variant for the cells to be inactive in relation to the ras or ras-like signal transduction pathway under the assay conditions in the absence of the ligand, and it being necessary in the latter variant for them to be inactive in relation to the ras or ras-like signal transduction pathway under the assay conditions in the presence of the ligand.

In an experimental system of the invention which is preferably used, the fusion protein comprises as effector section a mutated human Ras protein (Ha-Ras, L61) which lacks the farnesylation sequence which ensures membrane localization of the protein.

It is also possible for other protein components which,

besides the membrane receptor and, in particular, its mediator section and the effector protein, are themselves involved in the translocation of the effector protein or polypeptide directly or indirectly to be expressed where appropriate as the fusion proteins which do not occur naturally in the cell.

It may be added, for clearer understanding of the teaching of these documents, that the term "ligand" is intended to mean in the present context only those binding partners for receptors and, in particular, membrane receptors which elicit on binding to the ligand-binding section of such a receptor a structural change, in particular a conformational change and/or an enzymatic modification, e.g. by phosphorylation or dephosphorylation, whose effects on the mediator section result in an effector protein or polypeptide which is able to activate a Ras or Ras-like signal pathway in the cell binding to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors). This may comprise, in particular, structural and, specifically, conformational changes which take place in vivo on binding of a natural ligand and examples of which have been explained above. However, consideration is also given to cases in which the structural and, specifically, conformational change does not correspond, or corresponds only partially, to the structural or conformational change taking place on binding of a ligand which occurs in vivo in the cell. Binding partners which do not elicit such a structural change are not embraced by the term "ligand".

The terms "Ras and Ras-like signal pathway" or "signal pathway subsequent to a Ras protein", used synonymously herein, also embrace the so-called ras-like signal pathways which are controlled by various other members of the Ras family. Among the members of the Ras family there are ones which, despite originating from

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different organisms, are able to activate one and the same signal transduction pathway in the chosen target cell. One example thereof is the human Ha-Ras (L61) which is able also to activate a ras signal pathway in
5 *Saccharomyces cerevisiae* which acts on the cell cycle and whose activation is essential for reproduction of yeast cells. Other members of the Ras family are able only to activate a single signal pathway specific for them.

10

A number of members of the Ras family, such as the aforementioned Ha-Ras (L61), activates signal pathways which act on the cell cycle and whose activation are essential for cell reproduction via activation of
15 specific transcription factors. Other Ras proteins of this type activate signal pathways which specifically lead to activation in each case of one of a multiplicity of transcription factors which are specific for genes other than those of the cell cycle.
20 In the present context, a common feature of all ras signal pathways is that they require for their activation an active Ras protein present on the cell membrane, and the Ras protein requires for its activity where appropriate the simultaneous presence of a
25 guanine nucleotide exchange factor on the cell membrane.

Reference in these documents to an inactivation of a ras signal pathway or of a ras-like signal pathway
30 always means an inactivation of a level of the ras protein and/or of a guanine nucleotide exchange factor specific therefor. Said signal pathway inactivation occurs in a cell in the present context preferably only under certain environmental conditions, such as
35 temperature, and can thus be induced and abolished again by specific adjustment of environmental conditions.

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In the cellular context, a ras or ras-like signal transduction pathway is activated in each of the explained embodiments by the action of the active signal transduction components. Use of a cell in which this ras or ras-like signal pathway is not activated in the absence of the membrane receptor indicated according to the invention, because of mutations, at least under certain conditions, it is possible to detect one such solely by the activation mediated [lacuna] the translocation, resulting from the ligand binding or, alternatively, resulting from the lack of ligand binding to precisely this membrane receptor, of the effector protein or polypeptide onto the cell membrane, via phenotypical changes, e.g. growth or gene or reporter gene activity, in the cell.

The effector protein or polypeptide is preferably able to activate ras signal transduction pathways which act on the cell cycle and whose activation is essential for cell reproduction. Alternatively and likewise preferably it acts on one of the Ras signal pathways which serves to activate transcription factors for genes which need not be essential for cell reproduction.

25

The effector protein or polypeptide may have the activity of an active and, in particular, of a constitutively active Ras protein. Constitutively active Ras proteins show activity irrespective of the presence of guanine nucleotide exchange factor molecules, which various other Ras proteins require for their activity. For this purpose, the effector protein or polypeptide may comprise, for example, the amino acid sequence of an active or constitutively active Ras protein which occurs in nature, e.g. the human Ha-Ras (L61), or of parts thereof. Or it may comprise amino acid sequences which are derived from such sequences, for example by attachment, exchange, modification, insertion or deletion of amino acids.

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An alternative possibility is for the effector protein or polypeptide to house the activity of a functional guanine nucleotide exchange factor. In this respect, the amino acid sequence of the effector protein or polypeptide may likewise comprise, for example, sequences of naturally occurring guanine nucleotide exchange factors or partial sequences thereof, or it may be derived therefrom, for example by attachment, exchange, modification, insertion or deletion of amino acids. In a preferred embodiment, the amino acid sequence of the effector protein or polypeptide is derived from the amino acid sequence of the CDC25 protein from *Saccharomyces cerevisiae*, of an SOS protein from a mammal or of an SOS-like protein derived from any organism.

In addition, the invention comprises DNA molecules which encode novel fusion proteins, to which the invention likewise relates, and vectors, in particular plasmids, cosmids, viral or phage genomes, which comprise at least one of these DNA molecules. Particular vectors of the invention are suitable for the transformation or transfection of host cells or for the expression of at least one fusion protein of the invention. For the latter purpose, a DNA molecule of the invention in the vector is under the control of a promoter which is capable of functioning in a host cell and which makes expression possible and controls it.

Preparation of the fusion proteins, DNA molecules and vectors of the invention can take place by protocols known in the prior art (see, for example, Sambrook, J., Fritsch, E.F., Maniatis, T. (1989), *Molecular Cloning. A Laboratory Handbook*, Cold Spring Harbor Laboratory, New York; *Current Protocols in Molecular Biology* (1991)). Even if the fusion proteins can in principle be prepared by complete synthesis from individual amino derivatives, for which various methods are available in

the prior art, they are normally produced by expression of the appropriate genes in cells. In this case, the gene for the fusion protein may be present extrachromasomally or integrated into the genome of the host cell. The cloning of the genes for the fusion proteins starting from known gene sections which encode protein sections with the necessary functions or else, in the case of the ligand-binding or receptor section, at present only presumed functions likewise form as part of the standard abilities of a skilled worker, such as the construction of vectors, such as transcription or transfection vectors or else expression vectors, in which the gene is present functionally linked to a promoter effective in the producing cell, the transformation or transfection of host cells as well as the cultivation of the transformed or transfected host cells for producing the protein. The isolation and purification of the fusion proteins of the invention can take place through use of conventional methods such as precipitation, use of various chromatographic methods such as gel filtration, affinity chromatography etc. Affinity chromatography in particular allows selective binding only of the fusion protein, for example on use of specific antibodies or binding proteins which are bound to the matrix and which are directed against a determinant of a section, which is heterologous in relation to the host cell, of the fusion protein. An alternative possibility is for the fusion protein to be expressed, for example, also as precursor protein which has an additional domain with a specific property of binding to a particular affinity column. After binding and subsequent elution from the affinity column it is then possible for the additional domain to be eliminated selectively from the precursor protein, which is now already in essentially pure form, to produce the fusion protein of the invention. However, where the additional domain has no effect on the suitability of the fusion protein for the assay of the invention, it is also possible

alternatively to dispense with the elimination step. One example of such a domain consists of a plurality of, for example 10, histidine residues additionally attached at the N terminus ("His tag") and specifically
5 binding to a metal chelate affinity chromatography column. Concerning all the techniques mentioned and the reagents necessary therefor, including vector molecules, reference may be made to standard literature (e.g. Sambrook, J., Fritsch, E.F., Maniatis, T. (1989),
10 loc. cit.; Current Protocols in Molecular Biology (1991)) and an immense number of individual protocols.

Another central aspect of the invention besides the cells explained above are also cells which comprise one
15 or more of the membrane receptors as described in claim 1, in particular one or more of the membrane receptor fusion proteins of the invention. Within the framework of this invention it is moreover possible for single, a plurality of or else all domains of the
20 membrane receptor/fusion protein and/or where appropriate also parts of one or more sections or domains of the latter to be heterologous in relation to the host or initial cell.

25 To prepare the cells of the invention it is possible, for example, to transform or transfect initial cells with an expression vector which contains a gene for a membrane receptor with the features explained in claim 1, in particular a fusion protein of the
30 invention under control of a promoter capable of functioning in the initial cell. Suitable initial cells are prokaryotic as well as eukaryotic cells. Examples of initial cells are, inter alia, bacterial cells, such as those of the genus *Escherichia* or *Bacillus*, for
35 example certain strains of *Escherichia coli* or *Bacillus subtilis*, yeast cells, such as certain strains *Saccharomyces cerevisiae*, insect cells, animal cells such as COS-7, vero, CHO cells, mouse myeloma cells, human FL cells etc.

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The cells can also be provided in the form of ghost forms of the yeasts lacking cell walls, which are likewise embraced by the term "cells" in the present
5 context.

An essential feature of the cells of the invention is that, when there is a lack of binding of ligand to the ligand-binding section of the membrane receptor, the
10 effector protein or polypeptide is not able to bring about the activation of the specific Ras or Ras-like signal pathway in the cells or, alternatively, that the effector protein or polypeptide is not able, specifically when there is ligand binding to the
15 ligand-binding section of the membrane receptor, to bring about the activation of the specific Ras or Ras-like signal pathway in the cells.

In a preferred embodiment of this invention, the cell
20 of the invention is characterized in that, in the absence of the membrane receptor set forth in claim 1, at least under certain conditions a Ras or Ras-like signal pathway in the cell cannot be activated and, in particular, the signal pathway which the effector
25 protein or polypeptide is able to activate. Thus, cells in which a particular ras signal transduction pathway is active or inactive depending on the temperature are known in the prior art. Cells of this type can be employed as initial cells for expression of the fusion
30 protein or membrane receptor of the invention.

The inactivation, which is present at least under certain conditions, of a ras signal transduction pathway results from a Ras protein and/or guanine
35 nucleotide exchange vector which is incapable of functioning at least under the particular conditions. The inactivation may derive from genetic mutation or complete or partial gene deletion. For example, a Ras protein intrinsic to a cell can be inactivated when its

membrane-localization signal, usually a farnesylation signal, is deleted. A mutation in this membrane-localization signal with the effect that binding of the Ras protein to the cellular membranes can no longer take place would have the same effect. One example of a cell with a temperature-dependently defective guanine nucleotide exchange factor is the *Saccharomyces cerevisiae* yeast strain cdc25-2 (Broder et al., 1998). In this strain the guanine nucleotide exchange factor is no longer active at a restrictive temperature of 33 to 37°C, typically 36°C, but is fully capable of functioning at a temperature of, for example, 25°C. Since the guanine nucleotide exchange factor cooperates in this yeast strain with a Ras protein which controls a ras signal transduction pathway which acts on the cell cycle and is therefore essential for cell growth, no reproduction of the cells of the yeast strain is detectable at a restrictive temperature.

In a manner analogous to yeast strains with a temperature-sensitive mutation of an SOS protein intrinsic to yeast (CDC25-2) it is also possible to employ a yeast strain with a temperature-sensitive mutation of a Ras protein intrinsic to yeast.

An alternative possibility for preparing the cells of the invention is, however, also to use a cell, i.e. yeast cell or mammalian cell, which is in fact able to express a wild-type or mutated but active CDC25/SOS protein or Ras protein, but in which the gene encoding this active CDC25/SOS protein or Ras protein is under the control of an inducible promoter through which expression of the gene can be switched on or off deliberately by choosing particular culturing conditions. Examples of inducible promoters which can be employed in this connection are the galactose promoter or parts thereof from yeast or other organisms. The skilled worker is aware of a large number of inducible promoters suitable for this purpose

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from a wide variety of organisms. It is also possible to employ hybrid promoters with suitable inducibility.

If the cell of the invention expresses an active
5 CDC25/SOS protein or an active Ras protein, it is possible in another preferred embodiment of the invention for the CDC25/SOS or Ras protein additionally to contain a modification through which the protein degradation in the cell is speeded up. This
10 modification may be, for example, a ubiquitin signal or another signal which ensures the preferred degradation of a protein modified in this way in the cell. The advantage of expressing a protein modified in this way during induction of the promoter is that degradation of
15 the CDC25/SOS or Ras protein produced at the time of induction of the promoter is speeded up after "switching off" of the promoter, i.e. after providing culturing conditions with which the promoter is not induced and accordingly there is no longer any
20 transcription of the CDC25/SOS or Ras gene. Accordingly, even a short time after "switching off" of the promoter it is no longer possible to detect any such active CDC25/SOS or ras protein in the cell. In the preferred situation where the mediator section of
25 the membrane receptor investigated according to the invention is able, via its effect on the effector protein or polypeptide, precisely to activate the signal pathway which is activated by the active CDC25/SOS or ras protein mentioned above, it is thus
30 possible even a short time after changing the culturing conditions to switch off the promoter for the activation of this signal pathway to be measured exclusively on the basis of the effects of the membrane receptor investigated according to the invention, which
35 may signify a considerable advantage in terms of time. It is additionally possible in this way to reduce significantly the background signal based on an activation, which is possibly still present to a small extent and is not attributable to the membrane receptor

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investigated according to the invention, of the signal pathway.

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If the inactivation or inactivatability of the ras
5 signal transduction pathway intrinsic to the cell is
based on a defect or absence of a guanine nucleotide
exchange factor, it is possible in the preferred case
where the effector protein or polypeptide is able to
activate precisely this ras signal transduction pathway
10 for this effector protein or polypeptide to have the
activity of a functional guanine nucleotide exchange
factor or of an active, in particular constitutively
active, Ras protein, each of which is able to activate
the inactive ras signal transduction pathway. If an
15 effector protein or polypeptide with an activity of a
non-constitutively active Ras protein is employed, it
is reasonable for it to have the following properties:
it requires activation by a different type of guanine
nucleotide exchange factor which is unable to interact
20 functionally with the Ras protein, intrinsic to the
cell, of the inactive ras signal transduction pathway.
It is possible where appropriate for this specifically
suitable guanine nucleotide exchange factor to be
coexpressed as heterologous factor in the cell or assay
25 cell of the invention.

If the inactivation or inactivatability of the ras
signal pathway intrinsic to the cell is attributable to
a defect or absence of a Ras protein intrinsic to the
30 cell, then in the preferred case where the effector
protein or polypeptide is able to activate precisely
this ras signal pathway the latter will have the
activity of an active, in particular constitutively
active, Ras protein. If the effector protein or
35 polypeptide has the activity of a non-constitutively
active Ras protein, then activation thereof preferably
takes place through a guanine nucleotide exchange
factor intrinsic to the cell, but, as an alternative to
this, it may also require a heterologous guanine

nucleotide exchange factor to be coexpressed in the cell.

5 The techniques of molecular biology necessary for preparing the cells of the invention, e.g. cloning, vector construction, transformation or transfection, selection of transformed or transfected cells and calculation of the transformed or transfected cells etc., are well known to the skilled worker, and many
10 general protocols exist for these purposes and may where appropriate require slight adaptation, see, for example, Sambrook, J., Fritsch, E.F., Maniatis, T. (1989), loc. cit.; Current Protocols in Molecular Biology (1991) and numerous protocols specifically
15 drawn up for a particular cell type. The expression of, for example, heterologous proteins and fusion proteins may moreover, as mentioned, take place starting from a gene present extrachromosomally within an episome, in particular plasmid, and take place starting from a gene
20 integrated into the genome of the initial cell. Various techniques are available to the skilled worker for producing cells in which a particular ras signal transduction pathway is inactivated at the level of the Ras protein and/or of a guanine nucleotide exchange
25 factor and, in particular, for specific gene inactivation which may also be necessary in another connection for certain assay situations, for example through antisense strategies or for targeted introduction of mutations or deletions in the
30 particular genes or relevant genome sections. In particular, there are various known possibilities for preparing cell mutants in which transcription of the genes for example for the Ras protein or a guanine nucleotide exchange factor can be inactivated
35 deliberately under certain conditions, e.g. temperature-dependently. For this purpose, these cells contain these genes in particular linked to promoters which become inactive under certain conditions, such as starting at a particular temperature.

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In a particular embodiment of the invention, the cells and assay cells of the invention are applied to a solid carrier. Suitable carrier substances known in the prior art are, in particular, polysaccharides, e.g. agarose, specific plastics such as polyacrylamides, polystyrene, polyvinyl alcohol, silicones, or else certain types of glass. The carrier may in this case be in the form of separate particles, for example beads, or of an essentially plate-like substrate, e.g. in the form of a microtiter plate. The covering of the carrier with the cells may be complete, as is usually the case for example with carrier beads, or else present on only parts or sections thereof, such as, for example only in the wells of a microtiter plate. In a preferred embodiment, the cells of the invention are immobilized on so-called biochips (Wolf et al. 1997 and 1998). Immobilized on a solid carrier and, in particular, biochips, the cells are employed in this form in particular in high-throughput methods for detecting and measuring membrane receptor-ligand interactions. Methods for immobilizing the cells on these carriers are known to the skilled worker. It is possible, depending on the chosen carrier type, for the cells to bind to the carrier without further measures. In this case, the solid carrier phase is incubated with an essentially homogeneous population of cells, resulting in adhesion thereof to the solid phase. An alternative possibility is for the immobilization also to take place for example by means of chemical reagents such as glutaraldehyde, formalin etc. Measures of these types are known to the skilled worker.

The cells and fusion proteins of the invention are the basis for a number of *in vivo* assay methods, to which this invention likewise relates.

The assay methods which can be implemented using the first variant, i.e. effector protein or polypeptide

translocation onto the cell membrane possible only when there is ligand binding to the ligand-binding section of the membrane receptor investigated according to the invention, and which are explained in detail

5 hereinafter can be used, *inter alia*

1. to determine the suitability of a test substance as ligand for a receptor and, in this case, in particular to carry out mass screens with ligand derivatives in order to test which derivatives are
10 able to bind to the wild-type ligand-binding section of the receptor,
2. to detect the presence of a particular ligand in a sample,
3. to determine the concentration of such a ligand in
15 a sample,
4. to detect whether a compound is able to alter a binding activity of a ligand-binding section of a receptor in relation to a ligand, that is to say to act as agonist, antagonist or inhibitor, and in
20 this case in particular to carry out mass screens for finding such agonistic or antagonistic compounds; and
5. to detect the ligand-binding function of a polypeptide or protein suspected of having such a
25 function for ligands of particular receptors; the polypeptides or proteins may be, in particular, novel ligand-binding sections, derived from natural receptors by mutation, of receptors whose ligand-binding function still needs confirmation;
30 in this connection it is possible in particular to carry out mass screens with such novel, mutated ligand-binding sections which [lacuna] for example in the form of a receptor mutant library which contains, in particular, receptor mutants with
35 randomly localized mutations in the ligand-binding section, in order to find novel artificial, functional ligand-receptor partners.

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A first assay is used to determine the suitability of a test substance as ligand for a receptor section or, synonymously, ligand-binding section of a receptor and comprises the following steps:

- 5 (a) contacting the test substance with cells of the invention under conditions with which a Ras or Ras-like signal pathway cannot be activated in the cells in the absence of the membrane receptor, where the membrane receptor present in the cells contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway,
- 10
- 15 (b) investigating whether activation of the Ras or Ras-like signal pathway has taken place.
- Detection of the activation of the Ras or Ras-like signal pathway indicates in this case the ability of the test substance to bind to the ligand-binding
- 20 section.

Another in vivo assay makes it possible to detect the presence of a ligand for a ligand-binding section of a receptor in a sample which possibly contains the latter, and is characterized by the following steps:

- 25 (a) contacting the test substance with cells of the invention under conditions with which a Ras or Ras-like signal pathway cannot be activated in the cells in the absence of the membrane receptor, where the membrane receptor present in the cells contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor is able to
- 30
- 35 activate the inactive Ras or Ras-like signal pathway,
- (b) investigating whether activation of the Ras or Ras-like signal pathway has taken place.

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In analogy to the former assay, detection of the activation of the Ras or Ras-like signal pathway indicates the presence of a ligand for the ligand-binding section of a receptor in the sample.

5

Preferred Ras or Ras-like signal pathways, which are referred to hereinafter alternatively also as only Ras signal pathways, are regarded in this connection as being, as explained, signal pathways which act on the cell cycle and whose activation is essential for cell reproduction. Alternative and equally preferred ras signal pathways serve to activate transcription factors for genes which need not be essential for cell reproduction.

10

15

Detection of Ras signal pathway activation thus preferably takes place in the assays of the invention indirectly, i.e. via phenotypical changes, in this case in particular cell reproduction or gene or reporter gene activity, in the cells.

20

If, accordingly, the cells employed for the assays are ones in which the inactive Ras or Ras-like is a signal pathway which acts on the cell cycle and whose activation is essential for cell reproduction, the steps (b) explained above for investigating whether the cells are capable of reproduction under the conditions mentioned comprise, and detection of the ability of the cells to reproduce indicates the ability of the test substance to bind to or the presence of a ligand for the ligand-binding section of the membrane receptor in the sample.

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If, alternatively, the cells employed for the assays are those in which the inactive Ras or Ras-like signal pathway is a signal pathway which acts on the activity of a transcription factor for a gene which is not necessarily essential for cell reproduction, it is possible in the simultaneous presence of a construct

comprising a binding site for the transcription factor, a minimal promoter cooperating therewith and a gene, under the control of the minimal promoter, for a reporter protein, to use detection of expression of the reporter gene through detection of the transcription or translation products thereof for establishing the activation of the ras signal transduction pathway and thus that ligand binding has taken place on the ligand-binding section of the membrane receptor. This is because only on activation of the ras signal transduction pathway is it possible for there to be activation of said transcription factor, which is subsequently able to activate, via binding to its binding site, the minimal promoter and thus makes expression of the reporter gene possible.

In a preferred embodiment, the reporter gene or protein is a gene or protein which is heterologous in relation to the assay cell and whose presence can be specifically detected only when expression of the synthetic promoter-reporter gene construct takes place because of activation of the specific ras signal pathway and the resulting activation of the specific transcription factor. If detection takes place not as direct detection of the transcription or translation product by means of nucleic acid probes or antibodies specific therefor, but takes place, for example, via the enzymatic activity of the translation product, it is necessary on use of enzyme-encoding genes to ensure beforehand that the assay cell used does not, before the transformation or transfection with the synthetic promoter-reporter gene construct, contain an enzymatic activity like that of the heterologous enzyme expressed on ligand binding. A corresponding statement applies to the other types of reporter protein.

An alternative possibility is also to employ a gene which is homologous in relation to the assay as reporter gene. Reporter gene expression as a result of

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a transcription factor activation which takes place only because of the activation, to be detected in the assay, of a Ras or Ras-like signal pathway in the cells will in this case lead to an increase in the amounts of reporter gene transcription product present in the cells and, where appropriate, also an increased amount of the report gene translation product, and these can be measured by means of comparative experiments without use of ligands, e.g. by Northern blotting or Western blotting.

If these two latter alternatives are chosen, it is necessary to know the particular transcription factor activated by the chosen ras signal transduction pathway, and the promoter section, or its sequence, cooperating with this transcription factor. In order to make this assay variant possible, the assay cell will be transformed or transfected with a construct comprising the promoter functionally linked to the reporter gene, which can, where appropriate, take place by cotransformation or cotransfection together with one or more construct(s) which contain genes for other components of the assay system, e.g. the membrane receptor.

As mentioned, these constructs may, after transformation or transfection of an initial cell, be present chromosomally or extrachromosomally, i.e. as constituent of an episome, e.g. plasmid, in the assay cell.

At present, a number of ras signal transduction pathways, e.g. in different eukaryotic organisms, have already been completely researched in relation to the transcription factors activated thereby and the promoter regions cooperating therewith. A number of possibilities is thus available to the skilled worker for a selection in this regard.

In a preferred embodiment of the invention, the reporter protein comprises a modification which results in faster breakdown or degradation of the protein in the cell. This modification may be, for example, a ubiquitin signal or other signal which ensures breakdown of a protein modified in this way. The advantage of the use of a reporter protein which is broken down faster in an assay cell is obvious in the light of fact that a low background expression of the reporter gene construct will virtually always be detectable under the assay conditions, even without activation of the Ras or Ras-like signal pathway through the membrane receptor in the assay cell: the fast breakdown of the reporter protein significantly reduces the signal resulting from this background expression on detection of the protein level, i.e. of the reporter protein, because there is no accumulation of reporter protein over time. If, however, expression of the reporter protein is specifically activated by ligand binding to the membrane receptor and, resulting therefrom, activation of a Ras or Ras-like signal pathway, unambiguous detection is possible because the background signal is low. Since the half-life of the reporter protein in the assay cell will always be sufficiently long, detection of the reporter protein, produced as a result of ligand binding to the membrane receptor, will not be impaired in any way through the fast breakdown thereof.

The molecular biology techniques necessary for the preparation of transformation or expression vectors which contain the reporter gene functionally linked to a suitable specific promoter, and the transformation or transfection of cells, e.g. cloning, vector construction etc., are well known to the skilled worker and numerous general protocols exist therefor and require, where appropriate, at most slight adaptation (see, for example, Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) loc. cit.; Current Protocols in

Molecular Biology (1991)). The addition or fusion to a reporter gene of a sequence section which encodes a signal section which brings about faster breakdown of the expressed reporter protein, e.g. a ubiquitin
5 signal, is well within the capacity of a skilled worker.

The skilled worker is aware of numerous genes which can be employed as reporter genes in this connection and
10 which encode proteins which are amenable to simple and rapid detection. Examples thereof are genes which encode enzymatically active proteins, e.g. β -galactosidase, fluorescent proteins, e.g. GFP (green fluorescence protein) or chemoluminescent proteins.
15 Another possibility comprises genes which encode proteins which can be detected using specific antibodies. In this case, the antibody carries a detectable label or can in turn be detected by a secondary, labeled antibody. Possibilities of these
20 types are well known in the prior art. As already explained above, besides the necessity for detectability, it is essential only that the event to be detected in the cell, e.g. enzymatic activity, antibody binding, fluorescence, chemoluminescence, is
25 undetectable in the absence of the construct with the gene for the reporter protein.

An alternative possibility is for the transcription of the reporter gene to be detected on the basis of the
30 mRNA formed, by Northern blotting using probes specific therefor.

Another *in vivo* assay permits quantitative determination of the concentration of a ligand for a
35 ligand-binding section of a receptor in a sample which contains the latter, characterized by the following steps:

(a) contacting an aliquot of the sample with cells of the invention under conditions with which, in the

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absence of the membrane receptor, a Ras or Ras-like signal pathway in the cell cannot be activated, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose

5 binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in Claim 1, is able to activate the inactive Ras or Ras-like signal pathway,

10 (b) detecting quantitatively the extent of the activation of the Ras or Ras-like signal pathway by direct or indirect means,

(c) measuring the concentration of the ligand in the sample by comparing the measured extent of activation with corresponding values measured for known standard

15 concentrations of the ligand.

If cells for which the ras signal transduction pathway which is inactive at least under certain conditions is a signal pathway which acts on the cell cycle and whose

20 activation is essential for cell reproduction are used for quantitative detection of step (b), this takes place in a simple manner by determining the reproduction of the cells at a fixed time or the rate of reproduction of the cells under said conditions. The

25 resulting data are then compared with data obtained on the basis of standard preparations of known concentration, and the concentration of the sample is determined by calculation.

30 An alternative possibility in this case too is to detect quantitatively the extent of ras signal pathway activation on the basis of the extent of the expression in the cell of a reporter gene which is preferably heterologous to the cell. As explained above,

35 expression thereof is possible only because of the activation, brought about as a result of the activation of the signal pathway connected to a Ras protein, of a specific transcription factor. With this detection variant, the assay preferably takes place under

conditions which preclude cell reproduction, for example by using the cdc25-2 yeast mutant at restrictive temperatures, so that the transcribed amount of transcription product of the reporter gene or the expressed amount of reporter protein at a particular time or, alternatively, the expression rate of this reporter gene or protein can be determined with an essentially constant number of cells. However, the quantitative determination may also take place under proliferation conditions if, at the same time, the number of cells is determined continuously or at defined time intervals, and the values found for reporter gene expression are converted into values per unit value of the number of cells.

An alternative possibility in this case too is for quantitative detection to take place via expression of the reporter gene homologous to the host cell, in which case the increase in reporter gene expression observable at any time compared with the expression level present in cells without activation of the ras signal pathway is used to determine the result or value from each individual test, which is then compared with the values for the other tests to determine the overall result, the ligand concentration.

Another alternative *in vivo* assay makes it possible to detect whether a compound is able to alter a binding activity of a ligand-binding section of a receptor in relation to a ligand, that is to say to act as agonist, antagonist or inhibitor. This assay is characterized by the following steps:

(a) contacting the ligand in the presence of the compound with cells of the invention under conditions with which in the absence of the membrane receptor a Ras or Ras-like signal pathway in the cells cannot be activated, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component

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depends on the binding of a ligand to the ligand-binding section of the membrane receptor defined is able to activate the inactive Ras or Ras-like signal pathway.

- 5 (b) investigating whether and, where appropriate, to what extent activation of the Ras or Ras-like signal pathway takes place,
- (c) comparing the result of the investigation in step (b) with a result of an investigation obtained when the
- 10 assay is carried out in the absence of the compound.
- In this case, an increased activation of the Ras or Ras-like signal transduction pathway in the presence of the compound indicates an agonist function of this compound, whereas a reduced or, where appropriate, even
- 15 completely absent activation indicates an antagonist or inhibitory function of the compound.

Step (a) may moreover comprise adding the compound before the ligand to the cells, it being possible where
20 appropriate to preincubate the compound with the cells, adding the compound separately but at the same time as the ligand to the assay cells, or mixing the compound beforehand with the ligand and, where appropriate, carrying out a preincubation of the two compounds, and
25 only then adding the mixture to the assay cells.

If the compound is a peptide, polypeptide or protein, the compound can also be prepared by expressing a gene coding therefor inside the cell itself. For this purpose, the cells can be transformed or transfected with an expression vector which contains such a gene. The means and methods necessary for this are well known to the skilled worker.

35 If the compound to be tested for its agonistic or
antagonistic effect is expressed in the assay cell,
then the expression of the gene coding therefor
preferably takes place under the control of a
constitutively active promoter and with use of cells in

which the Ras or Ras-like signal transduction pathway is inactivated only under the specific assay conditions. Such a system makes it possible to preclude expression of the compound in the cell on its own
5 causing changes which might falsify the result of the assay. To preclude this it is necessary to detect under nonrestrictive conditions and in the absence of ligand the activity of the ras signal transduction pathway intrinsic to the cell, which is inactivated under
10 restrictive conditions. Under nonrestrictive conditions and in the absence of ligand the membrane receptor is in an inactive state, so that the detectable activation of the ras signal transduction pathway indicates that the expression product does not interfere with any
15 component of the Ras or Ras-like signal transduction pathway and, in particular, not with the Ras protein or guanine nucleotide exchange factor specific therefor. It is possible in this way to preclude, or minimize the probability, that the expression product interacts with
20 the effector protein or with an adaptor protein which is necessary for binding of the effector protein to the membrane component, instead of with the ligand-binding section of the membrane receptor, so that the ability of the component to activate the Ras or Ras-like signal
25 transduction pathway is abolished.

A corresponding test of activation of the particular Ras or Ras-like signal transduction pathway under nonrestrictive conditions, in the presence of the
30 compound and in the absence of ligand is carried out analogously with a compound added to the assay cells from outside.

If the Ras or Ras-like signal transduction pathway
35 which can be inactivated under the assay conditions is one whose activation is essential for cell reproduction, simply the normal reproducibility of the cells is ensured under nonrestrictive conditions. If detection takes place by use of a reporter gene

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activity, it is necessary to detect this reporter gene activity under nonrestrictive conditions.

For the detection under the restrictive conditions of the assay in step (b) there is likewise the possibility, for example, of detecting the activation of the signal pathway connected to a Ras protein via the expression, which takes place where appropriate and only on the basis of the activation resulting from the activation of the signal pathway connected to a Ras protein, of a reporter gene heterologous to the cells. The detection of the extent of the activation of the Ras or Ras-like signal pathway which takes place on detection of this activation may comprise a quantitative determination in which the amount, present in the cells, of transcription or translation product (reporter protein) of the reporter gene is determined at a particular time or the reporter gene transcription rate or the reporter protein expression rate is determined under the conditions mentioned.

An alternative possibility is also only to analyze aliquots, i.e. equal volumes of the assay solutions which have been produced and treated identically, apart from the addition of the compound, preferably ensuring equal or essentially equal numbers of cells in these aliquots. In this case the level of reporter gene expression is not quantified absolutely, but only a relative comparison of the levels of expression in the two assays is made possible.

In the case where the comparison in step (c) reveals that stronger expression of the reporter gene occurs in the presence of the compound, the compound is to be assumed to have an agonistic effect, and in the case where the comparison in step (c) reveals that lower expression of the reporter gene occurs in the presence of the compound, the compound is to be assumed to have an antagonistic effect. Like the quantitative assay

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described above, this assay is also preferably carried out under conditions with which no reproduction of the cells occurs.

5 An alternative possibility is for the reporter gene employed for the detection also to be homologous to the assay cell, in which case the increase, observable in each instance, in the expression, i.e. transcription and/or translation, of the reporter gene compared with
10 the level of expression present in cells without activation of the Ras or Ras-like signal pathway is used to determine the result.

15 If the cells employed for the assay are ones in which the inactive signal pathway connected to a Ras protein is a signal pathway which acts on the cell cycle and whose activation is essential for cell reproduction, step (b) comprises to investigate whether and, where appropriate to what extent the cells are capable of
20 reproduction under the conditions mentioned. In the case where the comparison in step (c) reveals that greater cell reproduction occurs in the presence of the compound, the conclusion is that the compound has an agonistic effect, and in the case where the comparison
25 in step (c) reveals that less cell reproduction occurs in the presence of the compound, the conclusion is that the compound has an antagonistic effect.

30 Like all the assays within the scope of this invention, this assay is also suitable in particular for mass screening, in this case for compounds which are receptor agonists and antagonists.

35 An alternative possibility is for the assay also to be used as additional test for confirming the ligand-binding property of a novel, in particular synthetic, ligand-binding section or for confirming the suitability of a test substance as ligand for the ligand-binding section. If the ligand-binding section

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has a ligand-binding property or if the ligand is suitable for the ligand-binding section, it should be observed on use of a known agonist for the ligand employed for the first detection of the ligand property, or for the ligand-binding section that there is increased activation of the Ras or Ras-like signal pathway, and on use of a known antagonist for the ligand or the ligand-binding section that there is in return a reduced activation of the Ras or Ras-like signal pathway.

A further alternative *in vivo* assay makes it possible to detect whether a polypeptide or protein suspected of a ligand-binding function of a receptor in fact has this function. This assay comprises the following steps:

(a) contacting cells of the invention with the ligand under conditions with which in the absence of the membrane receptor a Ras or Ras-like signal pathway in the cells cannot be activated, where the ligand-binding section of the membrane receptor comprises the polypeptide or protein to be investigated or consists thereof, and where the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway,

(b) investigating whether an activation of the Ras or Ras-like signal pathway has taken place.

Detection of the activation of the Ras or Ras-like signal pathway indicates that the ligand-binding section of the membrane receptor and, accordingly, the polypeptide or protein to be investigated has a ligand-binding function of a receptor.

For example, the membrane receptor present in the cells may comprise a ligand-binding section which contains a ligand-binding section derived from a ligand-binding

section of a naturally occurring receptor by mutation, or consists thereof.

As previously, it is possible to detect the activation
5 of the Ras or Ras-like signal transduction pathway on
use of cells with which the Ras or Ras-like signal
transduction pathway which is inactivate at least under
certain conditions is a signal pathway which acts on
the cell cycle and whose activation is essential for
10 cell reproduction, on the basis of cell reproduction
which takes place where appropriate.

An alternative possibility is to detect the activation
of the Ras or Ras-like signal transduction pathway also
15 on the basis of the expression, which can be detected
where appropriate, of a reporter gene in the cells. As
explained, if the reporter gene is heterologous,
expression thereof takes place only on the basis of the
activation, resulting from the activation of the Ras or
20 Ras-like signal pathway, of a specific transcription
factor. In the case of a homologous reporter gene the
aim is to detect the increase in reporter gene
expression, e.g. on the basis of larger amounts of
transcription or translation product, compared with the
25 expression level without activation of the specific Ras
signal transduction pathway.

The assay methods which can be implemented using the
second variant, i.e. effector protein or polypeptide
30 translocation onto the cell membrane possible only when
there is a lack of ligand binding to the ligand-binding
section of the membrane receptor investigated according
to the invention, and which are explained in detail
hereinafter, can be used, inter alia, for

- 35 1. determining the suitability of a test substance as
ligand for a receptor and, in this case, in
particular carrying out mass screens with ligand
derivatives in order to test which derivatives are

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able to bind to the wild-type ligand-binding section of the receptor,

2. detecting the presence of a particular ligand in a sample,

5 3. detecting whether a compound is able to change a binding activity of a ligand-binding section of a receptor in relation to a ligand, that is to say to act as agonist, antagonist or inhibitor, and in this case in particular carrying out mass screens for finding such agonistic or antagonistic compounds;

10 4. detecting the ligand-binding function of a polypeptide or protein suspected of having such a function for ligands of particular receptors; in this case too it is possible for the polypeptides or proteins in particular to be novel ligand-binding sections, derived from natural receptors by mutation, of receptors whose ligand-binding function is yet to be confirmed; in this connection, it is possible in particular to carry out mass screens with such novel mutated ligand-binding sections which [lacuna] for example in the form of a receptor mutant library which contains in particular receptor mutants with randomly localized mutations in the ligand-binding section, in order to find novel artificial, functional ligand-receptor partners.

The aforementioned assays can take place essentially analogously to the assay variants explained previously, using the first variant, but in this case the detection of the activation of the Ras or Ras-like signal pathway indicates the absence of a ligand for the ligand-binding section of the investigated membrane receptor in the assay cell. The latter assays will accordingly usually comprise two detections, specifically one detection in the presence of (potential) ligand, in which case no activation of the Ras or Ras-like signal pathway will be detectable if the (potential) ligand

has a ligand-binding property for the ligand-binding section of the membrane receptor investigated according to the invention, and another detection in the absence of the (potential) ligand, in which case such an
5 activation will ordinarily be detected.

In the detection of whether a compound is able to change a binding activity of a ligand-binding section of a receptor in relation to a ligand, that is to say
10 to act as agonist, antagonist or inhibitor, the detection takes place in the simultaneous presence of ligand and compound, in particular via

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- a) the activation, which is possibly in fact detectable if the compound has an antagonistic or
15 inhibitory effect, of the Ras or Ras-like signal pathway, for example with subsequent determination of the ligand concentration necessary for complete inactivity of the Ras or Ras-like signal pathway, at a particular concentration of compound, or a
20 determination of the dependence of the signal pathway activation on the concentration of compound at a particular ligand concentration; or
 - b) the inactivity, which is complete even at a relatively low ligand concentration when the
25 compound has an agonistic effect, of the Ras or Ras-like signal pathway; in this case too it is possible to determine the dependence of the complete inactivity of the Ras or Ras-like signal pathway on the concentration of the compound
30 and/or of the ligand in particular.

In the detection of a ligand-binding function of a polypeptide or protein suspected of such a function for ligands of particular receptors, the detection of the
35 ligand-binding function takes place analogously via the inactivity of the Ras or Ras-like signal pathway in the presence of ligand when this signal pathway is active in the absence of ligand.

Detection of receptor-ligand interactions by means of the assay methods, cells and fusion proteins of the invention is not confined to eukaryotic cells as in vivo test system but can alternatively also take
5 place in prokaryotic cells or in yeasts lacking cell walls, called ghost forms of yeasts.

Depending on the membrane receptor employed and, in particular, depending on the mechanism which depends on
10 the mediator section of the membrane receptor and which eventually leads to a translocation of the effector protein or polypeptide onto the membrane, it may in some embodiments of the invention, as already mentioned hereinbefore, additionally be necessary to provide in
15 the assay cells further conditions which ensure that the detectable translocation event occurs exclusively if there is an interaction of a ligand or, alternatively, if there is a lack of interaction of a ligand with the ligand-binding section of this membrane
20 receptor. Association of the effector protein or polypeptide with membrane-associated cell components which (in the absence of the membrane receptor and of the cell components which cooperate specifically and exclusively with the latter in signal transduction)
25 naturally occur in these cells, irrespective of the state of activation and modification, must be precluded. The precondition for this is that all components directly and indirectly involved in the translocation of the effector protein or polypeptide
30 onto the cell membrane can be rendered capable of mediating or bringing about the binding of the effector protein or polypeptide to a component of the cell membrane only as a result of the activation of the membrane receptor which is to be specifically tested,
35 on the basis of the binding or, alternatively, lack of binding of a ligand to the ligand-binding section and, more accurately, only as a result of the structural change in the mediator section caused by this activation.

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An alternative possibility is for a cell to be selected as assay cell in which, before introduction of the genetic information for the membrane receptor and the adaptor, facilitator and/or effector proteins or polypeptides which are required where appropriate, none of these components naturally occurs, that is to say are heterologous to this initial cell, and no components of the same specificity and, where appropriate, activity which might replace the latter are intrinsically present in the cell either; in relation to the membrane receptor, this may also apply only to the mediator section. Or care must be taken that an initial cell which in an original state once contained these components, in relation to the membrane receptor where appropriate also only the specific mediator section, no longer contains these components because of genetic or other modification before introduction of the genetic information for the membrane receptor and the adaptor, facilitator and/or effector proteins or polypeptides which are required where appropriate. Explained comprehensively, this relates at least to the following components:

- 35 - the mediator section,
 - where appropriate the adaptor proteins or adaptor
 protein sections cooperating therewith where
 binding of the effector protein or polypeptide
 takes place to the latter or via the latter, and

for example in the case of alternative 1)c) explained herein, where appropriate a facilitator component which need not be directly spatially associated with the mediator section of the membrane receptor but is, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section, activated or modified by the latter and, for this reason, facilitates a secondary event on another component of the cell membrane, as a result of which the effector protein or polypeptide specifically binds to this component of the cell membrane, which is not associated with the mediator section of the membrane receptor; alternatively, said secondary event in the cell may be facilitated only if there is ligand binding or, alternatively, lack of ligand binding of the ligand-binding section of the membrane receptor. Thus, in the latter case, no interaction partners for these facilitators which are able to exert an activation effect corresponding to the membrane receptor on the facilitator(s) must be present in the cell.

If, consequently, a cell system in which the membrane receptor to be tested does not naturally occur is used, it may be necessary to express, besides the membrane receptor, other proteins in this cell system, in particular where appropriate adaptor and/or facilitator proteins or polypeptides.

Concerning the assay conditions, no particular general requirements are necessary. However, in the case of detection of cell reproduction taking place where appropriate, it can thus be taken that the medium used makes this possible in principle. Where it is possible to inactivate genes and/or promoters in the cells, as explained, by choosing particular assay conditions, and inactivation is also intended during the assay, these conditions such as, for example, a particular

restrictive assay temperature, in the case of cdc25-2 cells for example 33-37°C, should be maintained during the assay. The chosen reaction medium should moreover not interact with the test compound or the ligand which are added to the medium in any way to impair the assay.

The ligands which can be investigated and determined in all the assay methods explained above are naturally occurring substances such as odorants, flavorings, peptides, peptide hormones and proteins, in particular cytokines, neurotransmitters, non-protein- or -peptide-like hormones, in particular steroid hormones, vitamins, e.g. vitamin D, thyroxine or retinoic acid, as well as substances which do not occur naturally, e.g. synthetic derivatives of natural ligands or toxins/poisons, such as dioxin.

In the majority of cases, the ligand-binding section of the membrane receptor will have an extracellular localization. On use of ligand-binding sections in particular of nuclear receptors, however, there is also the possibility that the latter have an intracellular localization. Since the ligands of nuclear receptors are mainly small molecules with lower relative molecular mass and mainly hydrophobic nature, they diffuse without further measures into the assay cells of the invention in order to enter into binding therein with a ligand-binding section, which has an intracellular localization directly on the cell membrane, of the membrane receptor. If desired and/or necessary, however, the cells can be pretreated before the assay in a suitable way in order to make the outer cell membrane more permeable for the test compound or the ligand to pass through. One example thereof is the preparation of cell ghosts by, for example, enzymatic treatment of cells. Cell ghosts of this type and those prepared in another way, and cells with a cell wall modified in another way to increase the permeability

are also embraced by the term "cells" in the present context.

5 If the ligands to be tested are peptides, polypeptides
or proteins, the latter may also be expressed in the
assay cell starting from the nucleic acid constructs
which encode the latter and have been introduced into
the assay cell, in a special variant also non-
constitutively but under the control of an inducible
10 promoter; it is moreover possible for the constructs to
be present after introduction into the assay cell
chromosomally or extrachromosomally, i.e. as
constituent of an episome, e.g. plasmid. The contacting
of the assay cell with the ligand accordingly takes
15 place in the case of non-constitutive expression under
conditions with which expression of the ligand to be
tested is induced in the cell. The skilled worker is
aware of a large number of inducible promoters for this
purpose, which can be induced, for example, by
20 particular temperatures or chemical compounds. On use
of membrane receptors with ligand-binding domains with
an extracellular localization, care must additionally
be taken that ligands expressed in the assay cell are
also secreted from the assay cells into the
25 extracellular space. Various possibilities for this are
also known to the skilled worker.

It should in general be stressed that on addition of
the ligand to be tested to the assay cell from outside
30 constitutive expression is possible in the assay cell
of all the components cooperating in the assay system
of the invention, i.e. in particular the membrane
receptor to be investigated, as defined in claim 1, the
effector protein or polypeptide, any adaptor and where
35 appropriate facilitator proteins or polypeptides, and
all the components of the signal pathway which is
specifically activated by the effector protein or
polypeptide on binding thereof to one component of the
membrane and is connected to a Ras protein, which are

involved only in this specific signal pathway. On expression of the ligand to be tested in the assay cell it is possible in the first variant, in which an effector protein or polypeptide translocation onto the membrane is detectable only on binding of ligand to the ligand-binding section of the investigated membrane receptor, for all components of the assay system, now including the ligand, apart from one to be constitutively expressed in the assay cell. The assay system component(s) whose gene(s) is or are provided under the control of an inducible promoter is or are then expressed in particular only under assay conditions, i.e. on investigation of the possible activation of the ras signal transduction pathway, on the basis of the specific induction of the inducible promoter or promoters employed in each case. In the second variant, in which the effector protein or polypeptide translocation onto the membrane occurs only when there is a lack of ligand binding to the membrane receptor, on expression of the ligand in the assay cell its gene will always be provided under the control of an inducible promoter in order to make detection possible even in the absence of the ligand.

Detection of the activation of the Ras or Ras-like signal transduction pathway takes place in a manner familiar to the skilled worker depending on the detection strategy. If the cells are immobilized on a solid carrier during the assay, it may be necessary or helpful, in particular in the case of detection of a reporter gene activity or of a reporter protein, to solubilize the cells before the detection reaction, i.e. to detach them from the carrier and, where appropriate, also disintegrate them. The measures and reagents necessary for this purpose are also well known to the skilled worker.

The invention additionally provides kits for use in the assays of the invention, which make it possible, for

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In addition, this kit, as well as all following ones, may contain inter alia where appropriate also assay

buffers, reagents for detecting the phenotypical activation of the Ras or Ras-like signal transduction pathway in these cells and/or instructions for use.

- 5 An alternative kit for the aforementioned assay methods comprises at least two of the following components:
- (a) cells in which at least under certain conditions a Ras or Ras-like signal pathway cannot be activated;
 - (b) a nucleic acid vector into which is expressibly
10 inserted a DNA sequence which encodes a membrane receptor, as defined above, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of
15 binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);
 - (c) a nucleic acid vector into which is expressibly
20 inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides;
 - 25 (d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding, or, alternatively, lack of binding of a ligand to the
30 ligand-binding section of the membrane receptor, to bind to a component of the membrane.

Another alternative kit for the aforementioned assays makes it possible with a particular choice of the
35 components listed hereinafter in particular to prepare the assay cell with a membrane receptor which contains an individually desired ligand-binding section. The kit comprises at least two of the following components:

(a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;

(b) a nucleic acid vector which comprises, in suitable arrangement:

- 5 - a DNA section which encodes a membrane-localization signal of a membrane receptor as defined above in connection with the membrane receptor;
- a DNA section which encodes a mediator section of a membrane receptor as defined above in connection with
- 10 the membrane receptor; and
- a suitably arranged insertion site for functional insertion of a DNA sequence which encodes a ligand-binding section of a receptor,

where, after insertion of a DNA sequence for the

15 ligand-binding section, the nucleic acid vector comprises a complete expressible gene for a membrane receptor as defined above, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of

20 binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

(c) a nucleic acid vector into which is expressibly

25 inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where

30 appropriate via other proteins or polypeptides;

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding, or,

35 alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

In a specific embodiment it applies to the two latter kits that component (c) of the kit comprises a nucleic acid vector whose DNA sequence encodes an effector protein or polypeptide in the form of a fusion protein
5 composed of an effector section and of an adaptor protein or polypeptide which makes binding possible to a component of the membrane, where appropriate via other proteins or polypeptides.

10 In another specific embodiment of component (c) of the two latter kits, the nucleic acid vector present therein comprises a DNA sequence which encodes an effector protein or polypeptide in the form of a fusion
15 protein composed of an effector section and of an antibody or binding protein section with specific binding affinity for a tag or epitope structure which is present on the membrane receptor and becomes accessible for the antibody or the binding protein only because of conformational changes, where appropriate in
20 combination with enzymatic activity, resulting from binding of ligand or, alternatively, lack of binding of ligand to the ligand-binding section of the membrane receptor.

25 It also applies additionally to the two latter assays that the cells present in the kit may, for an intended detection of a reporter gene activity inter alia additionally also contain a construct comprising a binding site for a transcription factor whose
30 activation results from the activation of the specific Ras or Ras-like signal pathway whose activation is to be detected by the assay, a minimal promoter and the reporter gene as explained above, or it is possible alternatively to provide, separately from the cells, a
35 transformation or transfection vector with the transcription factor binding site-minimal promoter-reporter gene construct or with another type of construct comprising the transcription factor binding site and the minimal promoter and, in addition, a

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suitably arranged insertion site for a reporter gene which can be chosen freely.

5 The invention also provides kits for the assay methods of the invention for detecting whether a polypeptide or protein has a ligand-binding function of a receptor.

10 A kit suitable for this purpose comprises cells of the invention, and the membrane receptor present therein comprises a ligand-binding section comprising or consisting of a polypeptide or protein suspected of having a ligand-binding function of a receptor.

15 An alternative kit comprises at least two of the following components:

- (a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;
- 20 (b) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes a membrane receptor as defined above, where the ligand-binding section of the membrane receptor comprises a polypeptide or protein suspected of having a ligand-binding function of a receptor, or is
25 formed therefrom, and the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the
30 inactive Ras or Ras-like signal pathway in the cells mentioned under (a);
- (c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of
35 ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides;

- (d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding, or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

An alternative kit for use in said assay makes it possible, on specific selection of the components indicated hereinafter, inter alia for an assay cell to be equipped specifically with a membrane receptor which comprises as ligand-binding section a polypeptide or protein which is desired by the user of the kit and is to be investigated for its ligand-binding function. Such a kit comprises at least two of the following components:

- (a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;
(b) a nucleic acid vector which comprises, in suitable arrangement:

- a DNA section which encodes a membrane-localization signal of a membrane receptor as defined above in connection with the membrane receptor;
- a DNA section which encodes a mediator section of a membrane receptor as defined above in connection with the membrane receptor; and
- a suitably arranged insertion site for functional insertion of a DNA sequence which encodes a polypeptide or protein suspected of having a ligand-binding function of a receptor,

where, after insertion of a DNA sequence for the ligand-binding section, the nucleic acid vector comprises a complete expressible gene for a membrane receptor, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section, formed from the polypeptide or protein suspected of having a ligand-

binding function or a receptor of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

- (c) a nucleic acid vector into which is expressibly
5 inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where
10 appropriate via other proteins or polypeptides;
(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding, or,
15 alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

- It also applies to the two latter kits that, in a
20 specific embodiment, component (c) of the kit comprises a nucleic acid vector whose DNA sequence encodes an effector protein or polypeptide in the form of a fusion protein composed of an effector section and an adaptor protein or polypeptide which makes binding possible to
25 one component of the membrane, where appropriate via other proteins or polypeptides.

- Likewise, in another specific embodiment of component (c) of the two latter kits, the nucleic acid vector
30 contained therein comprises a DNA sequence which encodes an effector protein or polypeptide in the form of a fusion protein composed of an effector section and an antibody or binding protein section with specific binding affinity for a tag or epitope structure which
35 is present on the membrane receptor and which becomes accessible for the antibody or the binding protein only because of conformational changes, where appropriate in combination with enzymatic activity, resulting from binding of ligand to the ligand-binding section of the

membrane receptor or, alternatively, dissociation of the ligand of the ligand-binding section, i.e. lack of binding of ligand to the ligand-binding section of the membrane receptor.

5

If it is intended to detect a reporter gene activity, in one embodiment the cells in the aforementioned kits additionally contain a construct comprising a binding site for a transcription factor whose activation results from activation of the specific ras signal pathway whose activation is to be detected by the assay, a minimal promoter and the reporter gene, as explained above, or it is possible alternatively to provide, separately from the cells, a transformation or transfection vector with the transcription factor binding site-minimal promoter-reporter gene construct or with another type of construct comprising the transcription factor binding site and the minimal promoter and, in addition, a suitably arranged insertion site for a reporter gene which can be chosen freely.

It is additionally possible for all the kits of this invention in possible embodiments to comprise inter alia additionally at least one of the following components:

(b) where appropriate a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one facilitator protein which need not be directly spatially associated with the mediator section of the membrane receptor but is, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, activated or modified by this mediator section and, for this reason, facilitates a secondary event on another component of the cell membrane, as a result of which the effector protein or polypeptide specifically binds to this component of the cell membrane, which is not

associated with the mediator section of the membrane receptor;

(f) where appropriate reagents for the transformation or transfection of the cells with transformation or
5 transfection vector(s),

(g) where appropriate reagents for detection of the phenotypical activation of the signal transduction pathway connected to a Ras protein in these cells.

10 In a preferred embodiment of the invention, the kits of the invention comprise the cells immobilized on a solid carrier, as explained above, in particular on biochips (cf. Wolf et al. 1997 and 1998). Immobilization of the
15 cells on biochips, and in the individual wells of microtiter plates, is particularly suitable for mass screens and, in particular, high-throughput methods for detecting and measuring membrane receptor-ligand interactions, so that a plurality of separate assay methods can be carried out on such a plate. It is also
20 possible in this connection to provide different cells of the invention, i.e. in particular cells with different ligand-binding section, in wells in respectively defined sections on one and the same microtiter plate.

25 If the cells in the kit are immobilized on a solid carrier it may be necessary or helpful, in particular on detection of a reporter gene activity or transcription of a reporter protein, to solubilize the
30 cells before the detection reaction, i.e. detach them from the carrier and, where appropriate, also disintegrate them. In this case it is possible for the reagents mentioned under g) for detecting the phenotypical activation of the Ras or Ras-like signal
35 transduction pathway also to comprise suitable solubilizing reagents which, in particular, contain one or more surface-active agents or surfactants.

The invention also extends to

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- ligands for a binding section of a receptor,
- compounds which are able to alter a binding activity of a ligand-binding section of a receptor in relation to a ligand (referred to as "modifying compounds" hereinafter) and
- polypeptides or proteins having a ligand-binding function of a receptor,

which have been determined or found by means of one of the assay methods of the invention, and compositions containing these ligands, compounds and/or polypeptides or proteins.

In relation to polypeptides or proteins having a ligand-binding function of a receptor and having been derived from a naturally found or synthetically produced molecule for production of the membrane receptor as defined in claim 1, the invention comprises both the fragment which is present in the membrane receptors employed according to the invention and has a ligand-binding function, and the initial fragment or molecule. It may be remarked in relation to this, only for the sake of clarity, that the production of the membrane receptor usually takes place by expression of a nucleic acid sequence encoding this membrane receptor in a cell. A polypeptide or protein with ligand-binding function of a receptor is accordingly derived from a larger initial molecule usually in an analogous way at the nucleic acid level, by merely using one or more sections of the nucleic acid sequence encoding the initial molecule, where appropriate with subsequent cloning for attachment of sections which encode other membrane receptor components or sections, for expression of the membrane receptor. The deriving may also include one or more slight nucleic acid sequence modifications in the initial sequence or in the nucleic acid section(s), preferably of a type such that the resulting nucleic acid molecule still hybridizes under stringent conditions with the respective initial nucleic acid molecule.

The invention accordingly also comprises a method for identifying polypeptides or proteins, in particular receptors, which have a ligand-binding function of a

5 receptor, which comprises:

- preparing a cell of the invention with a membrane receptor having the features described in claim 1 and comprising the whole of such a polypeptide or protein or a part of such a polypeptide or protein
10 which presumably contains the sequence sections essential for the ligand-binding function, and
- using this cell to carry out the *in vivo* assay method of the invention for detecting whether a polypeptide or protein has a ligand-binding
15 function of a receptor.

and the molecules identified by this method.

The invention likewise extends to

- the use of the aforementioned ligands, modifying compounds and polypeptides or proteins, identified
20 by the assay methods of the invention, as pharmaceuticals, where appropriate after formulation with excipients and/or carriers customary in this sector, and
- the use of the ligands, modifying compounds and polypeptides or proteins as lead substances for
25 developing ligands, modifying compounds and polypeptides or proteins which are derived therefrom - in particular by derivatization - in
30 particular those with corresponding or improved activity compared with the respective lead substance.

Thus, the invention also comprises a method for
35 preparing ligands, modifying compounds, polypeptides or proteins by derivatization one or more times starting from ligands, modifying compounds, polypeptides or proteins identified by the assay methods of the

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invention. This method may comprise, where appropriate, additionally the steps of

- also testing the ligands, modifying compounds, polypeptides or proteins obtained by the derivatization, using the assay methods of the invention, for ligand function or ligand-binding function and/or
- formulating the ligands, modifying compounds, polypeptides or proteins obtained by the derivatization, as pharmaceutical in conventional way.

The invention further extends also to the ligands, modifying compounds, polypeptides and proteins obtained by this method, i.e. the functional derivatives obtained by this method.

For a use as gene therapeutic agents intended to bring about the expression of a polypeptide or protein which has a ligand-binding function of a receptor, preferably of a receptor, in particular in human or animal cells, the invention further comprises nucleic acid molecules which are obtained, starting from a polypeptide or protein, in particular receptor, identified by the assay, identification, screening or preparation methods of the invention, by a method which comprises the provision of the gene encoding the polypeptide or protein, or a part, which comprises at least the nucleic acid sequence sections essential for the activity of the encoded polypeptide or protein, of this gene, in essentially pure form, i.e. in particular essentially free of other nucleic acids which are unnecessary or even deleterious for use as gene therapeutic agent. This method may, if not yet known, require preceding identification of the gene which encodes this polypeptide or protein. In particular, the method may additionally also comprise the following steps:

- if not yet known, determination of the amino acid sequence of the polypeptide or protein, in particular receptor, and/or
- if not yet known, identification of the gene which encodes this polypeptide or protein, and determination at least of the sequence of the coding sections of this gene,
- where appropriate carrying out modifications in the resulting nucleic acid sequence, for example for adaptation of the codon usage to that of a desired recipient organism, for introducing mutations or deleting intron sequences, and
- formulation of the nucleic acid sequence which has been modified where appropriate in the form of a gene therapeutic agent.

In a currently preferably used experimental system of the invention, the yeast strain cdc25-2 is used as cell system which is inactive in a ras or ras-like signal transduction pathway. As explained, the Ras protein is non-functional (Broder et al., 1998) in these cells at a restrictive temperature of 33-37°C, typically 36°C, as a consequence of the absence of a functional guanine nucleotide exchange factor (GEF; "guanyl nucleotide exchange factor"). Activation of the ras signal transduction pathway which is non-functional in this system at restrictive temperatures can be caused under these conditions only via the translocation, which can be brought about by various measures as described, of an effector protein or polypeptide able to activate this ras signal transduction pathway onto the cell membrane and can be detected by the fact that the yeast cells are able to grow irrespective of the presence of a functional GEF protein at restrictive temperatures (33-37°C, typically 36°C). As explained, the translocation of the effector protein or polypeptide requires the presence of a suitable ligand for the membrane receptor specifically provided in the cell.

In this system there is use in particular of a fusion protein which comprises as effector section a mutated human Ras protein (Ha-Ras, L61) lacking the farnesylation sequence which ensures membrane
5 localization of the protein.

The examples which are indicated hereinafter by way of example are intended to illustrate the invention further.

10

1. Epidermal growth factor (EGF) interaction with the EGF receptor (Figure 3)

Material and Methods

15 The following two vectors are used to transform *Saccharomyces cerevisiae* cdc25-2 cells:

1. An expression vector for constitutive expression of the human EGF receptor protein.

20

2. An inducible expression vector with a galactose-inducible promoter (Gal 1) for expressing the adaptor-effector fusion protein consisting of the human constitutively active ras protein part (Ha-ras, L61)
25 lacking the so-called CAAX box (the farnesylation signal for membrane localization) (= effector section) and of the murine Grb2 protein part (= adaptor section).

30 Yeast growth and manipulation

Conventional yeast transformation and manipulation protocols (see, for example, Hill et al. (1991), NAR 19, 5791) were used. The cells were plated out either on a glucose minimal medium which contains the
35 necessary amino acids and nucleotides (20 mg/l histidine, 100 mg/l leucine, 20 mg/l tryptophan, 20 mg/l uracil, 10 mg/l adenine sulfate), 2% glucose, 0.5% NH_4SO_4 , 0.17% yeast extract and 4% agar, or on galactose medium (1.7 g/l yeast nitrogen without amino

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acids, 5 g/l ammonium sulfate, 30 g/l galactose (> 99%)
20 g/l D-raffinose, 20 g/l glycerol (100%), 30 g/l
Bacto Agar).

- 5 Replica platings are carried out with velvet replica
plater. After transformation with the two expression
vectors described above, the cells are plated out on
glucose plates and incubated at 25°C (non-restrictive
temperature) for 3-4 days. Various test media (with and
10 without EGF, or with synthetic protein fragments with
ligand activity - see Komoriya et al., 1984) are then
inoculated with in each case three independent clones
and incubated at 37°C for 2-3 days, and then the growth
of the yeasts in the various liquid media is detected.
15 Yeasts which grow in galactose medium at 37°C only in
the presence of EGF (culture of yeast ghost cells
lacking cell walls) or only in the presence of [S-
acetamidomethyl Cys^{20,31}]-EGF-(20-31), an active EGF
fragment, indicate functional ligand-receptor
20 interaction.

2. Angiotensin interaction with the angiotensin receptor (Figure 4)

25 Material and Methods

The following vectors are used to transform
Saccharomyces cerevisiae cdc 25-2 cells:

1. An expression vector for constitutive expression
30 of the rat AT1A angiotensin receptor (Condon et al.,
1997)
2. One or more expression vectors for constitutive
expression of the three rat G-proteins G α_{13} , G β_1 and G γ_3
35 (Macrez-Leprêtre et al., 1997).
3. An inducible expression vector (Ura selection
marker) with a galactose-inducible promoter (Gall) for
expression of the adaptor-effector fusion protein

consisting of phosphatidylcholine phospholipase C minus the domain with the membrane-localization signal (= adaptor section) from rat vascular myocytes (Macrez-Leprêtre et al., 1996) and the human constitutively active ras protein portion (Ha-ras L61) lacking the so-called CAAX box (the farnesylation signal for membrane localization) (= effector section).

Yeast growth and manipulation

This corresponds to Example 1. The test media used for the angiotensin receptor experiments were employed plus/minus angiotensin or plus/minus L-162,313, a functional non-protein ligand of the angiotensin receptor (Perlmann et al., 1995). Yeasts which grow after 2 to 3 days at 37°C in galactose medium only in the presence of angiotensin (culture of yeast ghost cells lacking cell walls) or only in the presence of the non-protein ligand L-162,313 indicate functional ligand-receptor interaction.

20

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NEW PATENT CLAIMS

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1. A cell comprising a membrane receptor which comprises a ligand-binding section, a membrane-localization signal and a mediator section, where only when there is binding or, alternatively, only when there is a lack of binding of a ligand to the ligand-binding section is a structural change brought about with effects on the mediator section to result in binding of an effector protein or polypeptide, which is capable of activating a Ras or Ras-like signal pathway in the cell, to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), characterized in that the effector protein or polypeptide which is capable of activating a Ras or Ras-like signal pathway is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding to the component of the membrane possible, where appropriate via other proteins or polypeptides (adaptors).

2. A cell as claimed in claim 1, characterized in that the effector section which is capable of activating a Ras or Ras-like signal pathway is a guanine nucleotide exchange factor (GEF) or an active protein from the Ras family.

30

3. A cell as claimed in claim 2, characterized in that the effector section which is capable of activating a Ras or Ras-like signal pathway is the CDC25 protein from *Saccharomyces cerevisiae* or is derived from such a protein.

35

4. A cell as claimed in claim 2, characterized in that the effector section which is capable of activating a Ras or Ras-like signal pathway is an SOS

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protein from a mammal or an SOS-like protein from any organism, or is derived from such a protein.

5. A cell as claimed in any of claims 1 to 4,
5 characterized in that the fusion protein required enzymatic modification before it can be bound to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and in that an enzymatic activity necessary for the enzymatic
10 modification is activated only because of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section.

6. A cell as claimed in any of claims 1 to 5,
15 characterized in that the membrane receptor is a transmembrane receptor, an enzyme-coupled receptor, a G-protein-coupled receptor, a 7-transmembrane receptor or an odor receptor (or olfactorial receptor).

7. A cell as claimed in any of claims 1 to 5,
20 characterized in that the membrane receptor is a non-naturally occurring, synthetic membrane receptor.

8. A cell as claimed in claim 7, wherein the ligand-
25 binding section is derived from a ligand-binding section of a naturally occurring receptor.

9. A cell as claimed in any of claims 1 to 7,
30 characterized in that the ligand-binding section comprises the ligand-binding section of a transmembrane receptor, of a G-protein-coupled receptor, of a 7-transmembrane receptor, of an odor receptor (or olfactorial receptor) or of a nuclear receptor, or is derived from the latter.

35 10. A cell as claimed in claim 7, characterized in that the ligand-binding section is derived from a ligand-binding section of a naturally occurring receptor and, in particular, from the ligand-binding

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section of a transmembrane receptor, of a G-protein-coupled receptor, of a 7-transmembrane receptor, of an odor receptor (or olfactorial receptor) or of a nuclear receptor, by mutation, in particular by substitution, deletion, insertion and/or modification of one or more amino acids or groups of amino acids, or is a synthetic ligand-binding section generated by molecular modeling.

11. A cell as claimed in any of claims 1 to 10, characterized in that the mediator section comprises the cytoplasmic part of a G-protein-coupled receptor, sections thereof or an amino acid sequence derived therefrom.

12. A cell as claimed in claim 11, characterized in that the effector protein or polypeptide which is capable of activating a Ras or Ras-like signal pathway in the cell is in the form of a fusion protein of an effector section with an adaptor protein which can interact after the α subunit has dissociated off the heterotrimeric G-protein with the regions, which are freely accessible because of this, of the β and γ subunits of the G-protein which are associated with the mediator section, or with the α subunit of the heterotrimeric G-protein after dissociation off from the β and γ subunit of the G-protein or as a result of the dissociation of the heterotrimeric G-protein with the mediator section of the membrane receptor.

13. A cell as claimed in claim 12, characterized in that the fusion protein comprises an effector section and a GRK2 kinase or a GRK3 kinase.

14. A cell as claimed in claim 12, characterized in that the fusion protein comprises an effector section and an antibody which specifically recognizes and binds the β and γ subunits of the G-protein after the α subunit has dissociated off.

15. A cell as claimed in any of claims 12 to 14, characterized in that the effector section of the fusion protein comprises the sequence of an active Ras protein.

5

16. A cell as claimed in claim 11, characterized in that the mediator section comprises the cytoplasmic part of a G-protein-coupled receptor, sections thereof or an amino acid sequence derived therefrom, where the
10 G-protein interacting with the cytoplasmic part of the G-protein-coupled receptor activates in the activated state a phosphatidylinositol 3-kinase (PI3K).

17. A cell as claimed in claim 16, characterized in
15 that the effector protein or polypeptide which is capable of activating a Ras or Ras-like signal pathway in the cell is in the form of a fusion protein of an effector section with an Src homology 2 (SH2) or a pleckstrin homology (PH) domain.

20

18. A cell as claimed in any of claims 1 to 10, characterized in that the mediator section is able, as a result of the ligand binding or, alternatively, lack of ligand binding to the ligand-binding section, to
25 bind one or more adaptor proteins via which the effector protein or polypeptide which is capable of activating a Ras or Ras-like signal pathway in the cell, in the form of a fusion protein of an effector section with an adaptor protein or polypeptide section
30 which makes binding possible to the component of the membrane via one or more of the adaptor proteins, can bind to the mediator section.

19. A cell as claimed in claim 18, characterized in
35 that the mediator section is put in a position, as a result of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section, of exerting an enzymatic activity.

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20. A cell as claimed in claim 19, characterized in that the mediator section is put in a position, as a result of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section, of exerting a tyrosine kinase activity, a serine/threonine kinase or phosphatase activity.

21. A cell as claimed in claim 20, characterized in that the mediator section comprises the cytoplasmic part of the EGFR (epidermal growth factor receptor) or is derived from the latter.

22. A cell as claimed in claim 18, characterized in that the ligand-binding section is put into a position, as a result of a binding or, alternatively, lack of binding of a ligand for this ligand-binding section, of activating a separate, receptor-specific enzyme.

23. A cell as claimed in claim 22, characterized in that the separate, receptor-specific enzyme is heterologous to the cell.

24. A cell as claimed in claim 22 or 23, characterized in that the separate receptor-specific enzyme is a kinase and, in particular a tyrosine kinase.

25. A cell as claimed in any of claims 18 to 24, characterized in that the adaptor proteins Gbr2 or Shc can be bound by the mediator section as a result of the ligand binding or, alternatively, lack of ligand binding to the ligand-binding section.

26. A cell as claimed in any of claims 1 to 25, characterized in that the cell is a prokaryotic or eukaryotic cell.

27. A cell as claimed in claim 26, characterized in that the cell is a eukaryotic cell and, in particular,

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a yeast cell, specifically a yeast cell lacking cell walls.

28. A cell as claimed in any of claims 1 to 27,
5 characterized in that it is applied to a solid carrier.

29. A cell as claimed in claim 28, characterized in
that the cell is immobilized on biochips or enclosed in
microchambers.

10

30. A cell as claimed in any of claims 1 to 29,
characterized in that in the absence of the membrane
receptor at least under certain conditions a Ras or
Ras-like signal pathway in the cell cannot be
15 activated.

31. A cell as claimed in claim 30, characterized in
that the activatability of the Ras or Ras-like signal
pathway is temperature-dependent in the absence of the
membrane receptor.
20

32. A cell as claimed in claim 31, characterized in
that the lack of activatability of the Ras or Ras-like
signal pathway in the absence of the membrane receptor
25 above a particular temperature is derived from at least
one mutation of a guanine nucleotide exchange factor
intrinsic to the cell, which has the effect that the
latter is incapable of functioning above the particular
temperature.

30

33. A cell as claimed in claim 32, characterized in
that the cells are cells of the *Saccharomyces*
cerevisiae yeast strain cdc25-2 or are derived
therefrom.

35

34. A cell as claimed in claim 31 or 32, characterized
in that the lack of activatability of the Ras or Ras-
like signal pathway in the absence of the membrane
receptor above a particular temperature is derived from

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at least one mutation of a Ras protein intrinsic to the cell, which has the effect that the latter is incapable of functioning above the particular temperature.

5 35. An *in vivo* assay for determining the suitability of a test substance as ligand for a ligand-binding section of a receptor, characterized by the following steps:

10 (a) contacting the test substance with cells as claimed in any of claims 30-34 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or
15 polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 1, is able to activate this Ras or Ras-like signal pathway,

20 (b) investigating whether activation of the Ras or Ras-like signal pathway has taken place, where detection of the activation of the Ras or Ras-like signal pathway indicates the ability of the test substance to bind to the ligand-binding section.

25 36. An assay as claimed in claim 35, where step (b) comprises detecting the activation of the Ras or Ras-like signal pathway via reporter gene expression which takes place where appropriate and only because of the
30 activation, resulting from the activation of the Ras or Ras-like signal pathway, of a specific transcription factor, where detection of the expression of the reporter gene indicates the ability of the test substance to bind to the ligand-binding section.

35 37. An assay as claimed in claim 35, where in step (a) cells in which the inactive or inactivatable Ras or Ras-like signal pathway is a signal pathway which acts on the cell cycle and whose activation is essential for

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cell reproduction are employed, and step (b) comprises investigating whether the cells are capable of reproduction under said conditions, where detection of the ability of the cells to reproduce indicates the ability of the test substance to bind to the ligand-binding section.

38. An *in vivo* assay for determining the suitability of a test substance as ligand for a ligand-binding section of a receptor, characterized by the following steps:

(a) contacting the test substance with cells as claimed in any of claims 30-34 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 1, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether an activation of the Ras or Ras-like signal pathway has taken place,

(c) investigating cells employed in step (a) under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, for activation of the Ras or Ras-like signal pathway in the absence of the test substance, where detection of the activation of the Ras or Ras-like signal pathway in the absence of the test substance and the inactivity of the Ras or Ras-like signal pathway in the presence of the test substance indicates the ability of the test substance to bind to the ligand-binding section.

39. An assay as claimed in any of claims 35 to 38, characterized in that the test substance is a naturally occurring substance and, in particular, an odorant,

flavoring, peptide, peptide hormone, protein, in particular cytokine, growth factor, neurotransmitter, non-protein- or -peptide-like hormone and/or a vitamin.

5 40. An assay as claimed in any of claims 35 to 38, characterized in that the test substance is a non-naturally occurring substance and, in particular, a synthetic derivative of a natural ligand or a poison, in particular dioxin.

10

41. An assay as claimed in claim 40, characterized in that the test substance is employed as fusion protein comprising a presumed ligand domain.

15 42. A screening method for unknown ligands of a particular receptor, characterized in that an assay method as claimed in any of claims 35 to 38 is employed for the screening.

20 43. An *in vivo* assay for detecting the presence of a ligand for a ligand-binding section of a receptor in a sample which possibly contains the latter, characterized by the following steps:

(a) contacting the sample with cells as claimed in any
25 of claims 30-34 under conditions with which a Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding
30 to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 1, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether activation of the Ras or
35 Ras-like signal pathway has taken place, where detection of the activation of the Ras or Ras-like signal pathway indicates the presence of a ligand for the ligand-binding section of a receptor in the sample.

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44. An assay as claimed in claim 43, where step (b) comprises detecting the activation of the Ras or Ras-like signal pathway via reporter gene expression which
5 takes place where appropriate and only because of the activation, resulting from the activation of the Ras or Ras-like signal pathway, of a specific transcription factor, where detection of the expression of the reporter gene indicates the presence of a ligand for
10 the ligand-binding section of a receptor in the sample.

45. An assay as claimed in claim 43, where in step (a) cells in which the inactive or inactivatable Ras or Ras-like signal pathway is a signal pathway which acts
15 on the cell cycle and whose activation is essential for cell reproduction are employed, and step (b) comprises investigating whether the cells are capable of reproduction under said conditions, where detection of the ability of the cells to reproduce indicates the
20 presence of a ligand for the ligand-binding section of a receptor in the sample.

46. An *in vivo* assay for detecting the presence of a ligand for a ligand-binding section of a receptor in a
25 sample which possibly contains the latter, characterized by the following steps:

(a) contacting the sample with cells as claimed in any of claims 30-34 under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated
30 in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the
35 membrane receptor, as defined in claim 1, is able to activate this Ras or Ras-like signal pathway,

(b) investigating whether an activation of the Ras or Ras-like signal pathway has taken place,

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(c) investigating cells employed in step (a) under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, for activation of the Ras or Ras-like signal pathway in the absence of the sample, where a detection of the activation of the Ras or Ras-like signal pathway in the absence of the sample and the inactivity of the Ras or Ras-like signal pathway in the presence of the sample indicates the presence of a ligand for the ligand-binding section of a receptor in the sample.

47. A screening method for unknown ligands of a particular receptor in a sample, characterized in that an assay method as claimed in any of claims 43 to 46 is employed for the screening.

48. An *in vivo* assay for the quantitative determination of the concentration of a ligand for a ligand-binding section of a receptor in a sample which contains the latter, characterized by the following steps:

(a) contacting an aliquot of the sample with cells as claimed in any of claims 30-34 under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 1, is able to activate this Ras or Ras-like signal pathway,

(b) detecting quantitatively the extent of the activation of the Ras or Ras-like signal pathway by direct or indirect means,

(c) measuring the concentration of the ligand in the sample by comparing the measured extent of activation

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with corresponding values measured for known standard concentrations of the ligand.

49. An assay as claimed in claim 48, characterized in that the quantitative detection of the extent of activation of the Ras or Ras-like signal pathway in step (b) takes place indirectly by determining the amount present in the cells of a transcription or translation product of a reporter gene whose expression takes place only because of the activation, resulting from the activation of the Ras or Ras-like signal pathway, of a specific transcription factor, at a particular time or the expression rate of this reporter gene based on the transcription or translation product under said conditions, and in step (c) the measurement of the concentration of the ligand in the sample takes place by comparing the measured values with corresponding values measured for known standard concentrations of the ligand.

50. An assay as claimed in claim 48, characterized in that in step (a) cells in which the inactive or inactivatable Ras or Ras-like signal pathway is a signal pathway which acts on the cell cycle and whose activation is essential for cell reproduction are employed, and the quantitative detection of the extent of the activation of the Ras or Ras-like signal pathway in step (b) takes place indirectly by determining the reproduction of the cells at a fixed time or the reproduction rate of the cells under said conditions, and in step (c) the measurement of the concentration of the ligand in the sample takes place by comparing the measured values with corresponding values measured for known standard concentrations of the ligand.

51. An *in vivo* assay for detecting whether a compound is able to alter a binding activity of a ligand-binding section of a receptor in relation to a ligand, characterized by the following steps:

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5 (a) contacting the ligand in the presence of the compound with cells as claimed in any of claims 30 to 34 under conditions with which in the absence of the membrane receptor the Ras or Ras-like signal pathway in the cells cannot be activated, where the membrane receptor contains said ligand-binding section, and the effector protein or polypeptide whose binding to a membrane component depends on the binding of or, alternatively, the lack of binding of a ligand to the ligand-binding section of the membrane receptor, as defined in claim 1, is able to activate this Ras or Ras-like signal pathway,

10 (b) investigating whether and, where appropriate, to what extent activation of the Ras or Ras-like signal pathway takes place,

15 (c) comparing the result of the investigation in step (b) with a result of an investigation obtained when the assay is carried out in the absence of the compound.

20 52. An assay as claimed in claim 51, characterized in that step (b) comprises detecting the activation of the Ras or Ras-like signal pathway via reporter gene expression which takes place where appropriate and only because of the activation, resulting from the activation of the Ras or Ras-like signal pathway, of a specific transcription factor, and the quantitative detection, which takes place where appropriate, of the extent of the activation of the Ras or Ras-like signal pathway comprises determining the amount, present in the cells, of transcription or translation product of the reporter gene at a particular time or the expression rate of this reporter gene based on the transcription or translation product under said conditions, and in the case where the comparison in step (c) reveals that stronger expression of the reporter gene occurs in the presence of the compound, an agonistic effect of the compound is indicated, and in the case where the comparison in (c) reveals that

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lower expression of the reporter gene occurs in the presence of the compound, an antagonistic effect of the compound is indicated.

5 53. An assay as claimed in claim 52, characterized in that it is carried out under conditions with which no reproduction of the cells occurs.

10 54. An assay as claimed in claim 51, where in step (a) there is use of cells in which the inactive Ras or Ras-like signal pathway is a signal pathway which acts on the cell cycle and whose activation is essential for cell reproduction, and step (b) comprises investigating whether and, where appropriate to what extent, the
15 cells are able to reproduce under said conditions, and in the case where the comparison in step (c) reveals that greater cell reproduction occurs in the presence of the compound, an agonistic effect of the compound is indicated, and in the case where the comparison in step
20 (c) reveals that less cell reproduction occurs in the presence of the compound, an antagonistic effect of the compound is indicated.

25 55. An *in vivo* assay for detecting whether a polypeptide or protein has a ligand-binding function of a receptor, characterized by the following steps:

(a) contacting cells as claimed in any of claims 30 to 34 with the ligand under conditions with which in the absence of the membrane receptor, as defined in
30 claim 1, a Ras or Ras-like signal pathway in the cells cannot be activated, where the ligand-binding section of the membrane receptor comprises the polypeptide or protein to be investigated or consists thereof, and where the effector protein or polypeptide whose binding
35 to a membrane component depends on the binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway,

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(b) investigating whether an activation of the Ras or Ras-like signal pathway has taken place, where detection of the activation of the Ras or Ras-like signal pathway indicates that the ligand-binding section of the membrane receptor and, accordingly, the polypeptide or protein to be investigated has a ligand-binding function of a receptor.

56. An assay method as claimed in claim 55, characterized in that the ligand-binding section of the membrane receptor present in the cells is derived from a naturally occurring receptor section by mutation.

57. An assay as claimed in claim 55 or 56, where step (b) comprises detecting the activation of the Ras or Ras-like signal pathway via reporter gene expression which takes place where appropriate and only because of the activation, resulting from the activation of the Ras or Ras-like signal pathway, of a specific transcription factor, where detection of the expression of the reporter gene indicates the ligand-binding function of the ligand-binding section of the membrane receptor and, accordingly, of the polypeptide or protein to be investigated.

58. An assay as claimed in claim 55 or 56, where in step (a) cells in which the inactive or inactivatable Ras or Ras-like signal pathway is a signal pathway which acts on the cell cycle and whose activation is essential for cell reproduction are employed, and step (b) comprises investigating whether the cells are capable of reproduction under said conditions, where detection of the ability of the cells to reproduce indicates the ligand-binding function of the ligand-binding section of the membrane receptor and, accordingly, of the polypeptide or protein to be investigated.

59. An *in vivo* assay for detecting whether a polypeptide or protein has a ligand-binding function of a receptor, characterized by the following steps:

- (a) contacting cells as claimed in any of claims 30 to 34 with the ligand under conditions with which in the absence of the membrane receptor, as defined in claim 1, a Ras or Ras-like signal pathway in the cells cannot be activated, where the ligand-binding section of the membrane receptor comprises the polypeptide or protein to be investigated or consists thereof, and where the effector protein or polypeptide whose binding to a membrane component depends on the lack of binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway,
- (b) investigating whether an activation of the Ras or Ras-like signal pathway has taken place,
- (c) investigating cells as employed in step (a) under conditions with which the Ras or Ras-like signal pathway in the cell cannot be activated in the absence of the membrane receptor, for activation of the Ras or Ras-like signal pathway in the absence of ligands, where a detection of the activation of the Ras or Ras-like signal pathway in the absence of the ligand and the inactivity of the Ras or Ras-like signal pathway in the presence of the ligand indicates that the ligand-binding section of the membrane receptor and, accordingly, the polypeptide or protein to be investigated has a ligand-binding function of a receptor.

60. A kit for use in an assay as claimed in any of claims 35 to 54, which comprises cells as claimed in any of claims 30-34.

SIPPEL, Albrecht, E. et al.

07 February 2001

NEW PATENT CLAIMS 61 - 70

- 5 61. A kit for use in an assay as claimed in any of
claims 35 to 54, which comprises components (a) and (b)
indicated below and, where appropriate, additionally
one or both of components (c) and (d) indicated below:
- 10 (a) cells in which at least under certain conditions a
Ras or Ras-like signal pathway cannot be activated;
- (b) a nucleic acid vector into which is expressibly
inserted a DNA sequence which encodes a membrane
receptor, as defined in claim 1, where the effector
protein or polypeptide whose binding to a membrane
15 component depends on the binding or, alternatively,
lack of binding of ligand to the ligand-binding section
of the membrane receptor is able to activate the
inactive Ras or Ras-like signal pathway in the cells
mentioned under (a);
- 20 (c) a nucleic acid vector into which is expressibly
inserted a DNA sequence which encodes the effector
protein or polypeptide which, in the event of ligand
binding or, alternatively, lack of ligand binding to
the ligand-binding section of the membrane receptor, is
25 able to bind to a component of the membrane, where
appropriate via other proteins or polypeptides
(adaptors), and which is in the form of a fusion
protein of an effector section with an adaptor protein
or polypeptide which makes binding possible to the
30 component of the membrane, where appropriate via other
proteins or polypeptides (adaptors);
- (d) a nucleic acid vector into which is expressibly
inserted a DNA sequence which encodes at least one
adaptor protein, via which the effector protein or
35 polypeptide is able, when there is binding or,
alternatively, lack of binding of a ligand to the
ligand-binding section of the membrane receptor, to
bind to a component of the membrane.

62. A kit for use in an assay as claimed in any of claims 35 to 54, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

- 5 (a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;
(b) a nucleic acid vector which comprises, in suitable arrangement:

- a DNA section which encodes a membrane-localization signal of a membrane receptor, as defined in claim 1;

- a DNA section which encodes a mediator section of a membrane receptor, as defined in claim 1; and

- a suitably arranged insertion site for functional
15 insertion of a DNA sequence which encodes a ligand-binding section, as defined in claim 1,

where, after insertion of a DNA sequence for the ligand-binding section, the nucleic acid vector comprises a complete expressible gene for a membrane
20 receptor, as defined in claim 1, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor is able to activate
25 the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

(c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand
30 binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion
35 protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

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(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

63. A kit for use in an assay as claimed in any of claims 55 to 59, which comprises cells as claimed in any of claims 30-34, where the membrane receptor, as defined in claim 1, present therein comprises a ligand-binding section comprising or consisting of a polypeptide or protein suspected of having a ligand-binding function of a receptor.

64. A kit for use in an assay as claimed in any of claims 55 to 59, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

(a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;
(b) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes a membrane receptor, as defined in claim 1, where the ligand-binding section of the membrane receptor comprises a polypeptide or protein suspected of having a ligand-binding function of a receptor, or is formed therefrom, and the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under a);

(c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is

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able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

65. A kit for use in an assay as claimed in any of claims 55 to 59, which comprises components (a) and (b) indicated below and, where appropriate, additionally one or both of components (c) and (d) indicated below:

(a) cells in which a Ras or Ras-like signal pathway cannot be activated at least under certain conditions;
(b) a nucleic acid vector which comprises, in suitable arrangement:

- a DNA section which encodes a membrane-localization signal of a membrane receptor, as defined in claim 1;

- a DNA section which encodes a mediator section of a membrane receptor, as defined in claim 1; and

- a suitably arranged insertion site for functional insertion of a DNA sequence which encodes a polypeptide or protein suspected of having a ligand-binding function of a receptor,

where, after insertion of a DNA sequence for the ligand-binding section, the nucleic acid vector comprises a complete expressible gene for a membrane receptor, where the effector protein or polypeptide whose binding to a membrane component depends on the binding or, alternatively, lack of binding of a ligand to a ligand-binding section, formed from the

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polypeptide or protein suspected of having a ligand-binding function of a receptor, of the membrane receptor is able to activate the inactive Ras or Ras-like signal pathway in the cells mentioned under (a);

5 (c) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes the effector protein or polypeptide which, in the event of ligand binding or, alternatively, lack of ligand binding to the ligand-binding section of the membrane receptor, is
10 able to bind to a component of the membrane, where appropriate via other proteins or polypeptides (adaptors), and which is in the form of a fusion protein of an effector section with an adaptor protein or polypeptide which makes binding possible to the
15 component of the membrane, where appropriate via other proteins or polypeptides (adaptors);

(d) a nucleic acid vector into which is expressibly inserted a DNA sequence which encodes at least one adaptor protein, via which the effector protein or
20 polypeptide is able, when there is binding or, alternatively, lack of binding of a ligand to the ligand-binding section of the membrane receptor, to bind to a component of the membrane.

25 66. A kit as claimed in any of claims 60 to 65, in which the cells additionally contain a construct comprising a binding site for a transcription factor whose activation results from an activation of a specific Ras or Ras-like signal pathway whose
30 activation is to be detected by the assay, a minimal promoter and a reporter gene functionally linked thereto, where the minimal promoter is activated as a result of binding of the activated transcription factor to its binding site.

35 67. A kit as claimed in any of claims 60 to 65, characterized in that it additionally contains a transformation or transfection vector with a construct comprising a binding site for a transcription factor

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whose activation results from an activation of a specific Ras or Ras-like signal pathway whose activation is to be detected by the assay, a minimal promoter and a reporter gene functionally linked thereto, where the minimal promoter is activated as a result of a binding of the activated transcription factor to its binding site.

68. A kit as claimed in any of claims 60 to 65, characterized in that it additionally contains a transformation or transfection vector with a construct comprising a binding site for a transcription factor whose activation results from an activation of a specific Ras or Ras-like signal pathway whose activation is to be detected by the assay, a minimal promoter and an insertion site, suitably arranged for expression controlled by the minimal promoter, for insertion of a reporter gene, where the minimal promoter is activated as a result of a binding of the activated transcription factor to its binding site.

69. A kit as claimed in any of claims 60 to 68, which contains the cells immobilized or enclosed in microchambers of a solid carrier, in particular on biochips.

70. A method for identifying polypeptides or proteins, in particular receptors, which have a ligand-binding function of a receptor, which comprises:

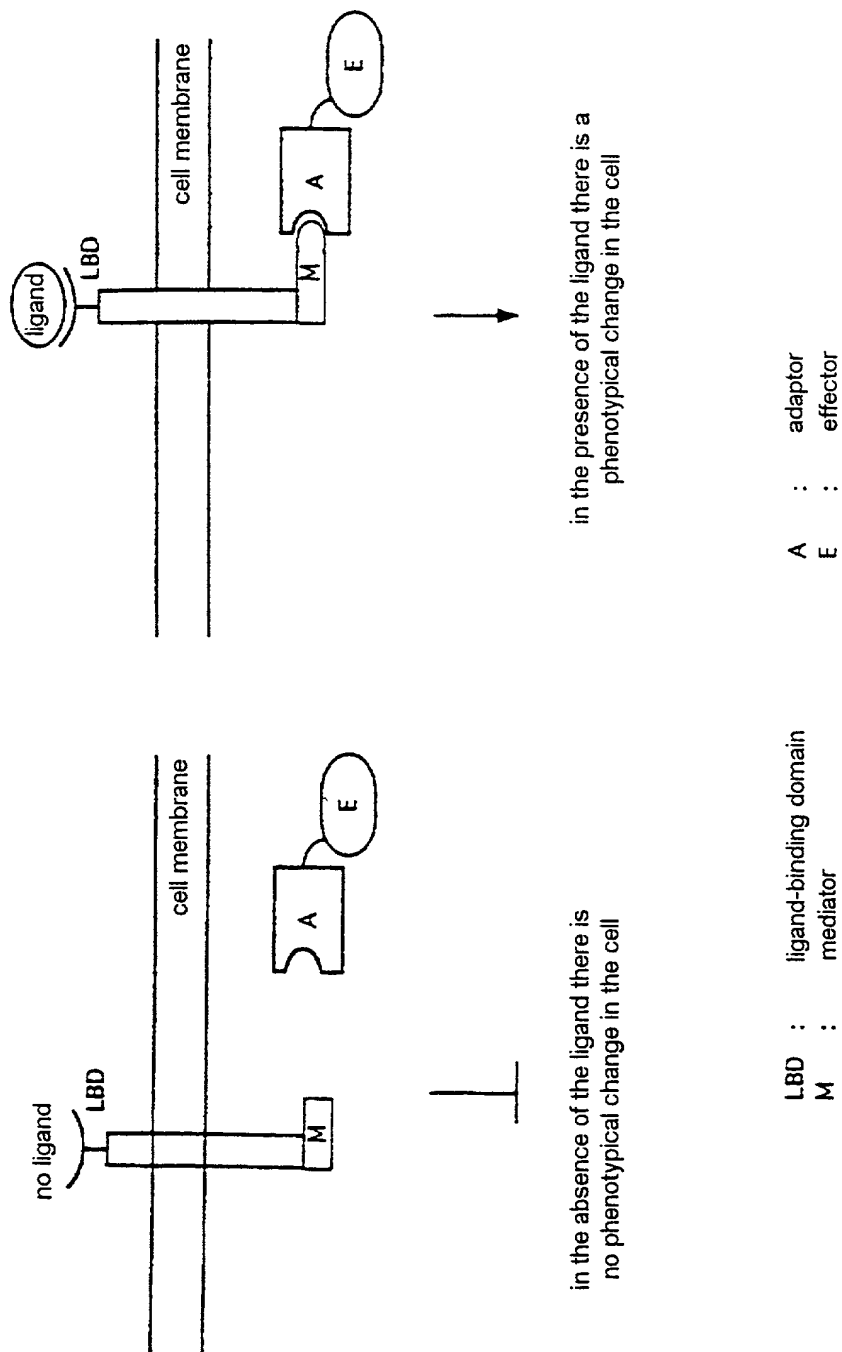
- preparing a cell as claimed in claim 1 with a membrane receptor having the features described in claim 1 and comprising the whole of such a polypeptide or protein or a part of such a polypeptide or protein which presumably contains the sequence sections essential for the ligand-binding function, and
- using this cell to carry out an *in vivo* assay method for detecting whether a polypeptide or

protein has a ligand-binding function of a
receptor, as claimed in any of claims 55 to 59.

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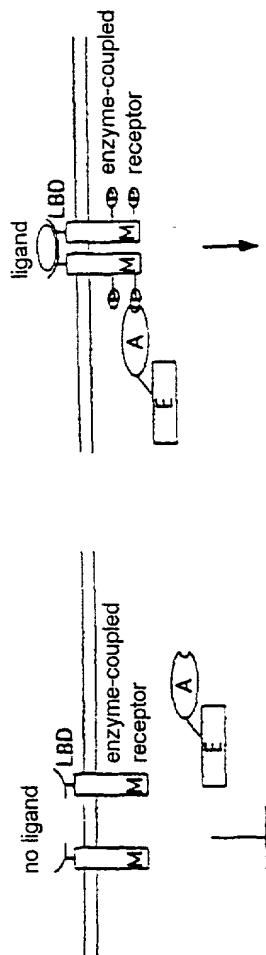
Diagrammatic representation of the detection of a ligand-membrane receptor interaction

Fig. 1



Diagrammatic representation of a ligand-membrane receptor interaction
of an enzyme-coupled and of a G-protein-coupled receptor

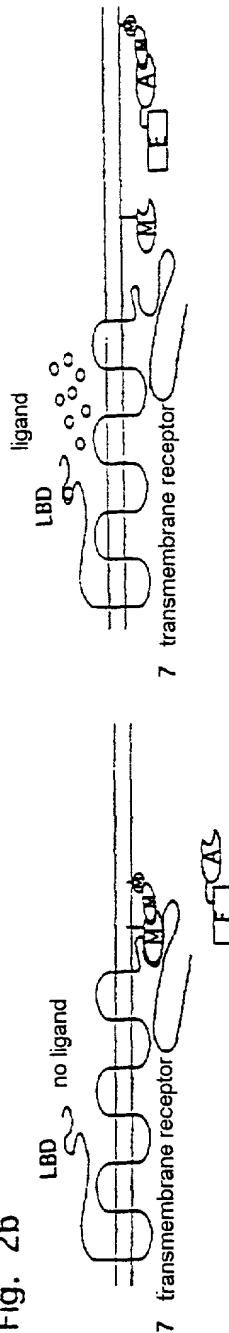
Fig. 2a



in the absence of the ligand there is
no phenotypical change in the cell

in the presence of the ligand there is a
phenotypical change in the cell

Fig. 2b



in the absence of the ligand there is
no phenotypical change in the cell

in the presence of the ligand there is a
phenotypical change in the cell

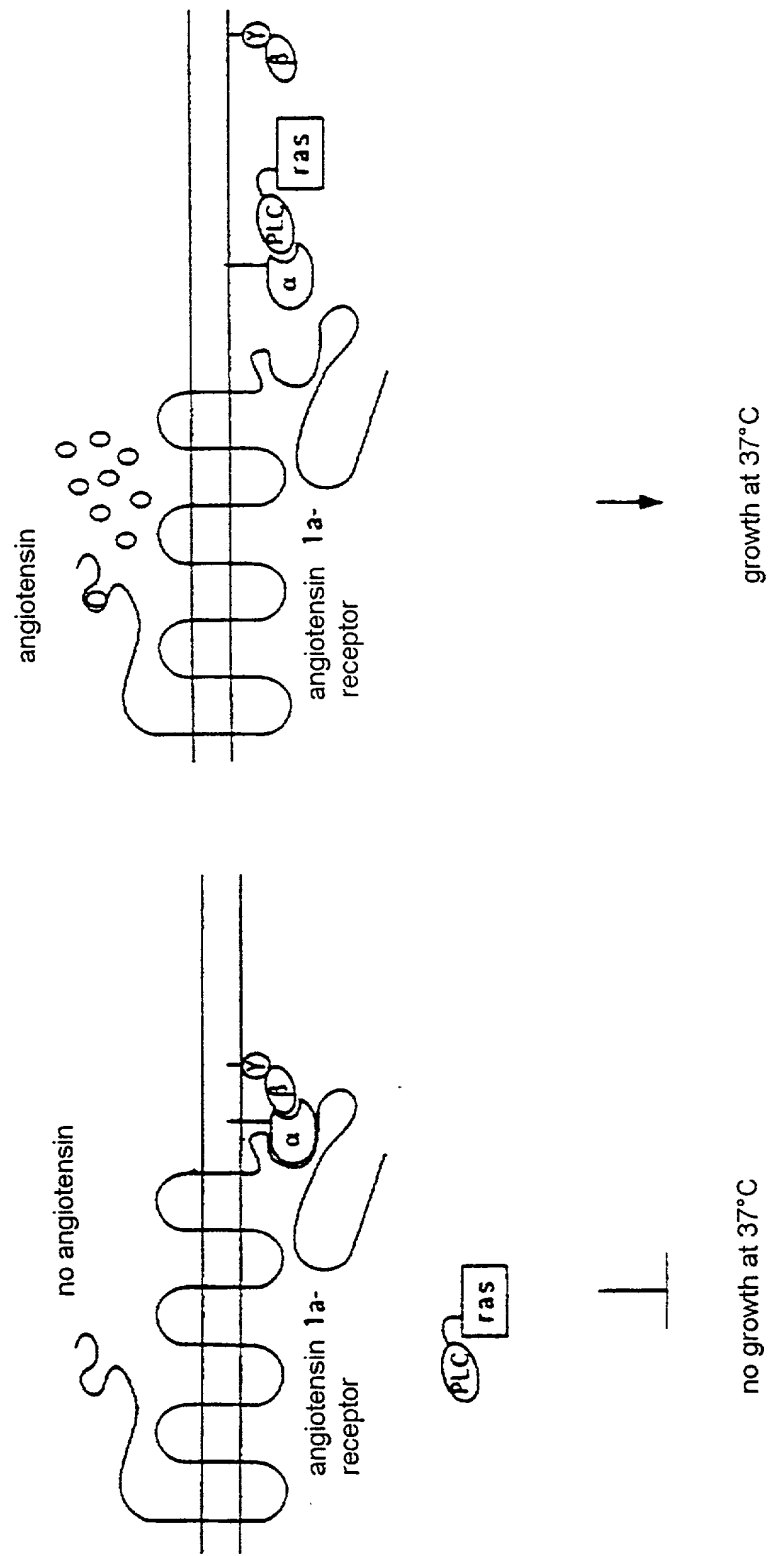
LBD : ligand-binding domain
M : mediator

A : adaptor
E : effector

Fig. 3



Fig. 4



COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
 (Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought of the invention entitled:

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application

Serial No. _____

on _____

and was amended

on _____ (if applicable).

☒ was filed as PCT international application

Number PCT/EP 99/10460

on December 29, 1999

and was amended under PCT Article 19

on February 12, 2001 and February 21, 2001 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
DE	198 60 833.0	Dec 30, 1998	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

Combined Declaration For Patent Application and Power of Attorney (Continued) <small>(Includes Reference to PCT International Applications)</small>				ATTORNEY'S DOCKET NUMBER PATWA 1	
I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:					
U.S. APPLICATION NUMBER		U.S. FILING DATE		PATENTED	PENDING
PCT APPLICATION NO.		PCT FILING DATE		U.S. SERIAL NUMBERS ASSIGNED (if any)	
POWER OF ATTORNEY: As a named inventor, I hereby appoint I. William Millen (19,544); John L. White (17,746); Anthony J. Zelano (27,969); Alan E.J. Branigan (20,565); John R. Moses (24,983); Harry B. Shubin (32,004); Brion P. Heaney (32,542); Diana Hamler-King (33,302); Richard J. Traverso (30,595); Richard E. Kurtz (33,936); John A. Sopp (33,103); Richard M. Lebovitz (37,067); John H. Thomas (33,460); Luan C. Do (38,434) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.					
Send Correspondence to: MILLEN, WHITE, ZELANO & BRANIGAN, P.C. Arlington Courthouse Plaza I, Suite 1400 2200 Clarendon Boulevard Arlington, Virginia 22201				Telephone No. (703) 243-6333 <u>235 99</u>	
				Direct Telephone Calls to: (703) 812-5311	
201	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
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	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY	
204	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY	
205	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY	
206	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY	

Combined Declaration for Patent Application and Power of Attorney (Continued)

(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER

207	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY
208	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY
209	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY
210	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY
211	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY
212	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	DATE	SIGNATURE OF INVENTOR 207	DATE
<i>X Edward E. Dine</i>	<i>X 18.06.01</i>		
SIGNATURE OF INVENTOR 202	DATE	SIGNATURE OF INVENTOR 208	DATE
<i>X Andre Dine</i>	<i>X 18.06.01</i>		
SIGNATURE OF INVENTOR 203	DATE	SIGNATURE OF INVENTOR 209	DATE
SIGNATURE OF INVENTOR 204	DATE	SIGNATURE OF INVENTOR 210	DATE
SIGNATURE OF INVENTOR 205	DATE	SIGNATURE OF INVENTOR 211	DATE
SIGNATURE OF INVENTOR 206	DATE	SIGNATURE OF INVENTOR 212	DATE